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TESIS DOCTORAL

Function and evolution of multiple signals in processes of sexual selection of Iberian green lizards (*Lacerta Schreiberi*)

Función y evolución de señales múltiples en procesos de selección natural del lagarto Verdinegro (*Lacerta Schreiberi*)

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Renáta Kopena

DIRECTORES

José María Rueda
Pilar López Martínez

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Memoria presentada por la licenciada Renáta Kopena para optar al grado de Doctor en Ciencias Biológicas, dirigida por el Doctor José Martín Rueda y la Doctora Pilar López Martínez del Departamento de Ecología Evolutiva del Museo Nacional de Ciencias Naturales-Consejo Superior de Investigaciones Científicas.

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Renáta Kopena

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El doctorando

Renáta Kopena

V°B° del Director

José Martín Rueda

V°B° del Director

Pilar López Martínez

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These were seven very long and tough years, full with unexpected obstacles, but finally I have finished...

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General Section

FUNCTION AND EVOLUTION OF MULTIPLE SIGNALS IN PROCESSES OF SEXUAL SELECTION OF IBERIAN GREEN LIZARDS (*LACERTA SCHREIBERI*)

Introduction

Mate choice is a basic process for sexually reproductive animals. Mate choice can be based on direct benefits, where males (or rarely females) offer resources to the other sex (e.g. parental care, "presents", etc.) (Hoelzer 1989), or indirect benefits, where only genetical stock is offered to females to increase future fitness thanks to their offspring's good genes (Zahavi 1975) and/or attractiveness (Fisher 1930). Males have to advertise their direct or indirect fitness benefits to females to be chosen for reproduction, thereby increasing their own fitness. These advertisements are the basis of sexual communication between males and females, which can be long-term stable if signaler's and receiver's benefits exceed the costs of communication. Signaling is time and energy consuming, furthermore, it can increase predation risk by increasing conspicuousness. However, signaling is worth if the animal can increase its fitness by being successful in mate acquisition. From the point of view of the receiver, the assessment of the signal needs time and may increase brain costs or require developing adequate sensory systems, but direct and/or indirect benefits can increase its own fitness too.

Informative signals are honest due to the costs of the signals. According to the **Zahavi's handicap principle** (1975), the signal system has to be costly for the signalers in a way that the production of a specific signal of high intensity is costlier for low quality individuals than for the good quality ones. If producing a signal of high intensity is costlier for the low quality males, then production of signals of high intensity will be worth only for high quality males. This differential cost allows to signal honestly a male's better quality and fitness, which will allow females to select a mate that will provide offspring of better quality

and higher fitness. However, signals do not have to be necessarily informative to be used in mate choice. In accordance with the **Fisherian runaway selection** (1930), both preferred male trait and female preference have genetical variability. Females may mate according to their preferences and initially the polymorphism of preference and of preferred trait too exist stable in the population and without evolutionary alteration. Initially, the average of the preferred male trait is close to the ecological optimum and female preference will be around this ecological optimum on average, because this ensures the highest survival for their offspring. But if the female preference changes in any direction from average, the evolution will be fast. Males with the preferred trait can fertilize more females and females with this preference have higher fitness by producing more attractive sons. Mate of females with this preference and males with this preferred trait cause a linkage disequilibrium and a strong positive feedback. The process moves on a self-generating way to spread of preference and the preferred trait fast increasing, so the selection runaway. The process will stop when reduced survival handicap from production or wearing cost of the trait exceed benefits of mate acquisition. Another possibility for signal evolution is the exploitation of **preexisting sensory bias** in the receiver (Ryan et al. 1990; Endler and Basolo 1998). Based on ecological or physiological reasons, females can be more sensitive to, for example, certain specific voice frequency or color wavelength, for which detection can be more effective. This can give the possibility to males of using these more detectable signals to elicit favorable reactions from females (e.g. special acoustical signals can elicit approaching from female túngara frogs, *Physalaemus pustulosus*). Males with the preferred signal take advantage against other males. Selection supports the spread of the signal and adaptation to sensory preferences of the receiver. However, signals resulting of the last two hypotheses are unreliable because they cannot indicate mate viability or direct benefits but take advantage of an optional preference or facilitated detection (Moller and Pomiankowski 1993), nevertheless these can cause higher fitness by having more attractive offspring (Candolin 2003). Furthermore, all sensory systems have biases, thereby evolving of mating biases are inevitable (Kirkpatrick and Ryan 1991; Arak and Enquist 1995). The preexisting sensory bias theory explains only the initial attraction of females. But traits evolved by exploiting

this sensory trap can be condition dependent or be affected by a runaway selection, because the maintenance of female preferences may require honesty of signal or the linkage disequilibrum between preferred male trait and female preference to increase fitness of females. For example, the chemosensory preference of Iberian rock lizard females for cholesta-5,7-dien-3-ol (provitamin D_3), a lipid found in both prey and males' scent, likely evolved by a preexisting sensory bias, but allocation by males of this chemical to femoral secretion as a signal is costly and only high quality males can afford it (Martín and López 2008). Thus, distinction and mutually exclusion of models explaining evolution of mate choice are likely not possible (Kokko et al. 2003).

Function and evolution of multiple signals in mate choice

The sexual displays that animals use in mate choice can be very complex, such as bright colorful ornaments, elaborated songs or voices and/or conspicuous courtship displays (Candolin 2003). There are several theories for the evolution and function of complex signals. Complex signals can consist of several components that can be only interpretable when they are perceived together (multicomponent signals), or signals where each signal can elicit a behavioral response on its own (multiple signals) and, in some special cases, where different types of signals can be detected by different sensory systems (multimodal signals) (Candolin 2003; Hebets and Papaj 2005).

Different signals can reflect different aspects of a potential partner's quality. These signals may be evaluated together and show the complete quality of an individual, or different receivers may concentrate on different signals and different aspects of quality according to their own quality or other traits. This is the **multiple messages hypothesis** (Wedekind 1992; Moller and Pomiankowski 1993; Johnstone 1997). For example, males of satin bowerbirds (*Ptilonorhynchus violaceus*) have ultraviolet plumage coloration which reflects feather growth rate, body size and level of blood parasite infection, while the quality of the built bower indicates ectoparasite load and body size (Doucet and Montgomerie 2003). Another typical case is when a receiver estimates overall quality of a signaler based on

multiple signals. In American goldfinch (*Carduelis tristis*), carotenoid-based yellow coloration reflects physical condition while the melanin-based black coloration rather shows the social status. The different information contents of different color signals can have their origin from different production costs (McGraw and Hill 2000). However, different signals can reflect the same quality (**Back-up signals hypothesis**) (Moller and Pomiankowski 1993; Johnstone 1997), which allow the receiver to estimate more accurately the signaler quality and make more difficult the cheat for the signaler. In females of the socially monogamous, biparental northern cardinals (*Cardinalis cardinalis*), body size and body condition strongly correlate with red-orange bill color and redness of underwing feathers (Jawor et al. 2004). Detectability and evaluation of signals can change depending on environmental conditions and distance, which can influence mate choice. Also, individuals can rely on different signals in different environmental conditions. Iberian wall lizards (*Podarcis hipanica*) use breeding coloration as long range signals and chemical signals for close range (López and Martín 2001). Usually the easiest valuable signal in all possible distances and environmental condition is the most emphasized one (Candolin 2003) (**Multiple sensory environment**, Candolin 2003). These signals can have a role as a backup for another one in case of environmental noise, for example changes in light levels or humidity. The efficacy of lizards' femoral secretion as chemical signals depends on humidity and temperature. More humid periods can increase growth of vegetation that can reduce the detectability of visual signals in the long range. Similarly, two different populations of *Anolis cristatellus* have two different dewlap colorations depending on which signal has better detectability in each different environment with different light intensity and spectral quality (mesic *vs* xeric habitat) (Leal and Fleishman 2004). Additional signals can be an alternative possibility for perception in case of spatial and/or temporal variability of the environment (**Efficacy backup hypothesis**).

Multicomponent signals can elicit stronger reactions in the receiver than a single signal due to a higher detectability, recognition, discrimination and memorability. The signaler can develop his signal by production of complementary components or non-informative accessory stimulus (**Receiver psychology hypothesis**). Many animals are

specialized in the detection of movement (e.g. Ewert and Hock 1972), likely because it signals danger or food. Vigorous movements frequently are parts of many displays which may exploit sensitivity of receiver for this fact, such as *Anolis* species using motion patterns of head and brightly colorful dewlap to communicate with conspecifics (Jenssen 1977; Fitch and Hillis 1984; Tokarz 1995), or in the human case, visual attractiveness of women and men equally increase when sex pheromones of the opposite sex are present too (Kirk-Smith et al. 1978).

Multiple signals also can evolve by Fisherian or unreliable signals that exploit arbitrary preferences or facilitated detection. Use of these kinds of signals by males can elicit female preference, but this mating is suboptimal for females and may select for female resistance for this trait. Males have to evolve new preferred traits and this process can lead to multiple ornaments. These traits cannot be used for long-time manipulation of female resistance, but they are necessary as a threshold before female attention turns to other traits (**chase-away sexual selection**) (Holland and Rice 1998). However, although female preference for unreliable multiple signals may seem neutral or maladaptive, it may have indirect benefits if the reproductive success of offspring increase due to the heritability of these manipulative traits (Cordero and Eberhard 2003; Kokko et al. 2003).

Components or signals of multiple displays can be related. If strength of preferences increases linearly with the number of concordant traits, the relationship is additive (Künzler and Bakker 2001), but generally the relationships between the strength of preference and number of signals are more complex and there are interactions (Candolin 2003). Females can attend to a cue depending on another one (**multiplicative interactions**) (Candolin 2003). Criterion of mate choice can be both the ornamental coloration and display rate of males in female guppies (*Poecilia reticulata*). In case of low intensity of display rates of two potential male mates, females decide based on coloration, but if both males have ornamental coloration, females prefer the male with higher display rate (Kodric-Brown and Nicoletto 2001). In some species, one cue is necessary to exceed a threshold value before attention is paid to other cues (**sequential use of cues**) (Candolin 2003). For example, in European

bitterling (*Rhodeus sericeus*), females have an initial choice decision based on male behavior and red coloration, but the final decision is based on the oviposition place (i.e. the quality of the live mussel that is defended by the male) (Candolin and Reynolds 2001). If the variation of a signal decreased, the importance of other signals can increase (Candolin 2003). Ipswich sparrows (*Passerculus sandwichensis princeps*) females prefer bigger territory size and song rate of males, but the primarily preferred trait is the one that has higher variance in that given year (Reid and Weatherhead 1990). Environmental changes can influence development of signals, which can give the opportunity to females to choose males based on that signal(s) that can better reveal the male quality under some given conditions. In lark bunting (*Calamospiza melanocorys*), females shift preferences on male traits across years to increase their reproductive success (Chaine and Lyon 2008). One cue can amplify or improve reception of another cue (Hasson 1989; Hasson 1990; Hasson 1997). For instance, the above-mentioned *Anolis* lizards increase dewlap movement detection by brightness coloration (Fleishman 2000). Male guppies (*Poecilia reticulata*) have a melanin-based black area around the carotenoid-based orange sexual signal that produces an illusion effect that enhances the orange area (Brooks 1996). A cue can affect the cost and expression of another cue (Johnstone 1996). In male red-billed streamertails (*Trochilus polytmus*), the asymmetry of the tail increases the cost of tail elongation (Evans et al. 1994).

Visual signals

Animals can communicate using color signals, developed armaments, behavioral displays or use all together in a complex signal. Coloration origins from pigments or from the structure of the integument. The most widespread and researched pigments are carotenoids and melanins, but pterins, flavonoids, porphirines and psittacofulvins are also important pigments. However, we have less information about their role as sexual signals.

Carotenoids are large fat-soluble hydrocarbons which only plants, algae, some bacteria and fungi can synthesize (Goodwin 1986), but no animals (except pea aphids, *Acyrthosiphon pisum*) (Moran and Jarvik 2010). Carotenoids have two major groups:

carotenes (β-carotene, lycopene) and xanthophylls (e.g. lutein, zeaxanthin). Carotenes consist of carbons and hydrogens only, while xanthophylls have at least one oxygen atom. Absorption and transportation of carotenoids need lipids in diet and from the body reserves too (lipid micelles, chylomicrons and lipoproteins) (Erdman et al. 1993). Different kinds of carotenoids have different efficacies of absorbance, and competition is also possible among these and other compounds (Kostic et al. 1995; Tyssandier et al. 2002, 2003), for example with vitamin E, because both carotenoids and vitamin E can use the same intestinal membrane transporter (Reboul et al. 2007). Absorbed carotenoids are able to transform to other carotenoids and derivatives by oxidation and reduction (Schiedt 1998). Carotenoids are capable to absorb light due to their conjugated bonds with highly delocalized π-electrons which reach excited state by comparatively low energy and correspond to light between 400-500 nm of wavelength producing yellow, orange or red coloration (Britton 1995).

Since animals cannot synthesize carotenoids de novo, they must obtain them from the diet. Several studies prove that carotenoid supplementation increases the expression of carotenoid-based signals in birds (Hill and Benkman 1995; Navara and Hill 2003) and fish (Baron et al. 2008; Pike et al. 2009) but interestingly, not in reptiles (Olsson et al. 2008; Steffen et al. 2010; Weiss et al. 2011). However, β-carotene can increase saturation of carotenoid-based ventral coloration in *Lacerta vivipara*, although not by carotenoid deposition, but rather by increasing background reflection (San-José et al. 2013). These results support that carotenoid-based sexual signals are limited by dietary carotenoids, that are very sensitive to variations in body condition and environmental conditions, and that their heritability is low (Badyaev and Hill 2000; Hadfield and Owens 2006).

Melanins are large polymers synthesized by sulfite-content amino acids (nonessential tyrosine and essential phenylalanin) (Hearing 1993) in melanosomes within melanocytes (Prota 2012). Melanin pigments are capable to absorb ultraviolet-visible light spectrum (Prota 2012). Two forms exist: eumelanins (with cystein) have black or grey colors, and pheomelanins (without cystein) have chestnut and buff colors. Melanins can deplete in the integuments (skin, hair and feathers). However, extracutaneous tissues, for

example neurons or phagocytes, can also accumulate melanin because these pigments have important roles in the immune and antioxidant systems (Riley 1992; Mackintosh 2001; Sugumaran 2002). Furthermore, melanins have important roles in tissue strengthening (Bonser 1995), photoprotection (in skin and eyes) (Ortonne 2002; Seagle et al. 2005; Reguera et al. 2014) and thermoregulation (Cloudsley-Thompson 1999; Reguera et al. 2014).

However, according to previous results, melanin-based coloration is strongly genetically correlated, has high heritability and is less sensitive to the variance of the environment and body condition (Hill and Brawner 1998; Roulin et al. 1998). Even so, current experiments show that melanin-based traits often associate with phenotypically plastic traits, for example behavior or physiology (including body condition), whose heritabilities are low (Hadfield and Owens 2006). The source or energy costs could be found in the background of this association. Extra melanin producing increases food intake rate in guppies (*Poecilia reticulata*) (Rodgers et al. 2013), and reduced availability of tyrosin and phenylalanin results in paler bibs in house sparrows (*Passer domesticus*) (Poston et al. 2005). However, the limits of access to these amino acids are not really known in natural conditions (Roulin 2015). Furthermore, the costs of synthesis of melanins are also little known. Some metals, such as calcium can increase melanin pigmentation in mammals (Prota 2012), they affect synthesis of melanin by enzyme cofactor of tyrosinase and may facilitate the molecular rearrangement of intermediate products (Solano and García-Borrón 2006), but these metals can have limited access in the nature (Klasing 1998; McDowell 2003). Maintenance of a melanin-based color badge can also be costly, mainly if the integument renews periodically, such as feathers or scales (Roulin 2015). However, melanin pigments can increase resistance against certain physically harmful agents (some parasites, ultraviolet radiation) (Bonser 1995; Kose and Møller 1999; Ruiz de Castañeda et al. 2012), although avoiding abrasion of the integument protective and cleaning capabilities may be necessary (Roulin 2015).

In several species, melanin-based badges are reliable status signals that only high quality individuals are able to afford (Santos et al. 2011; Diep and Westneat 2013).

Furthermore, more melanized individuals are more aggressive and more sexually active (Ducrest et al. 2008). The potential link between body condition (or mate quality) and melanin-based coloration may be caused by indirect reasons. For example, the linkage disequilibrium between melanogenic genes and genes that affect condition, or pleiotropical genes that can affect simultaneously to coloration and body condition (Roulin 2015). Although the relationship between mate quality and coloration is still unclear, melanin-based coloration has been suggested as a possible base for mate choice decisions in several species (e.g. Dale and Slagsvold 1996; Thusius et al. 2001; Kingma et al 2008).

Structural coloration is also widespread as a color source, but there have evolved very different structural elements in integument of animals to produce color by different kind of light scattering (reviewed in Simonis and Berthier 2012). Invertebrates have elaborated one or more dimensional phototonic structures, one or multilayered systems from chitin and air with chitinous components (e.g. Noyes et al. 2007; Simonis and Vigneron 2011). In birds, feathers usually have one or multiple layers of keratin (Cuthill et al. 1999; Doucet et al. 2006). However, sometimes barbules of feathers make more complicated this structure (Zi et al. 2003; Vigneron et al. 2006; Lee et al. 2010). Fish have leucophores which are dendritic cells with globular membranous vesicles that contain cytoplasm and purine crystals or colorless pteridines (Takeuchi 1976; Fujii 1993; Oliphant and Hudon 1993). Amphibians and reptiles have iridophores which contain reflecting purine crystals. These crystals are mostly made from guanin but sometimes are made with hypoxanthine or uric acid (Bagnara 1965; Fujii 1993). The relative spacing between each component and each other can cause different reflective effects. In case of a random crystal distribution, iridophores can reflect all wavelengths with white light or selectively reflect certain range of the spectrum (e.g., the Tyndall scattering crystals reflect short wavelength while transmit long wavelengths to the melanin layer that absorbs in this range, resulting in a non-iridescent light blue, so-called Tyndall blue) (Bagnara and Hadley 1973). In case of parallel arrangement of purine platelets, crystals reflect light selectively in a dominant range of the spectrum (Huxley 1968; Kinoshita and Yoshioka 2005) and can produce iridescence (Doucet and Meadows 2009). Color production of multilayer structures depends on the thickness and refractive index of the

purine crystal layers and the between positioned cytoplasm (Denton and Land 1971). This precise structure also defines the intensity of the produced color by constructive and destructive interference of different wavelengths of scattering light (Herring 1994; Kinoshita and Yoshioka 2005). The most common structural colors reflected maximally on short wavelengths resulting in color from ultraviolet to turquoise, but any color can be produced by light reflectance (Prum 2006).

Although it was previously accepted that the development of structural coloration do not possess biochemical or resource costs (Jawor and Breitwisch 2004), it has been found several times that structural coloration was affected by developmental stress (McGraw et al. 2002; Hill and Brawner 1998; Kemp et al. 2006; reviewed in Doucet and Meadows 2009) and condition (Keyser and Hill 1999; Fitzstephens and Getty 2000; Siefferman and Hill 2005; Peters et al. 2007), which, therefore, would allow a reliable indication of the signaler quality. Structural signals are widespread in the animal kingdom to advertise mate quality in invertebrates (Kemp 2008), fish (Siebeck 2004; Rick and Bakker 2008; Partridge and Cuthill 2010), amphibians (Secondi et al. 2012), reptiles (Bajer et al. 2010), and birds (Bennett et al. 1997; Hunt et al. 1998; Bitton et al. 2007), and to indicate male dominance in agonistic encounters in invertebrates (Shashar et al. 1996; Lim and Li 2006), fish (Siebeck 2004), reptiles (Stapley and Whiting 2006; Whiting et al. 2006; Bajer et al. 2011), and birds (Mateos and Carranza 1997; Alonso-Alvarez et al. 2004).

The dermal chromatophore unit of lizards

In the case of lizards, 3-4 contiguous cell layers are located under the epidermis and basal lamella whose interactions determine the lights reflected by the integument. These cell layers constitute the so-called 'dermal chromatophore unit' (Bagnara and Hadley 1973) (Fig. 1 a,b). In this unit, the top layer consists of xantophores and the interconnecting collagen. Xantophores absorb shorter wavelengths (violet-blue) depending on density of carotenoids, but longer wavelengths (or if carotenoids are lacking, short wavelengths too) are transmitted to the second layer, which is the layer of iridophores. Iridophores reflect the transmitted light

depending on their density and spacing. The remaining light reaches the melanophore layer where it is absorbed or, in case of low density of eumelanin (since lizard synthesize only eumelanin; Bagnara and Hadley 1973), light can be reflected also from the reflective shield of connective tissue that can be located directly beneath the melanin layer (Macedonia et al. 2000). Both thickness of pigment layers (Fig. 1c) and arrangement of reflective crystals can influence produced skin coloration (Fig. 1d) (Saenko et al. 2013). Furthermore, dispersion and aggregation of pigments by different covering of iridophores (especially in case of upward-extending dendritic melanophores) can influence reflecting lights of iridophores longer or shorter-wavelength light-reflecting response on skin coloration (Bagnara et al. 1968, Kasukawa et al. 1986, 1987; Nagaishi and Oshima 1989; Oshima and Kasai 2002). Although the newest researches show that the order of layers can change or that a layer can be doubled occupying a missing layer (Fig. 2d,e), the dermal chromatophore unit is a reliable scheme of lizard skin.

The color pattern of *L. schreiberi* depends on the absorbance and reflectance properties of different chromatophore layers in different body parts (Fig. 1a). Iridescent ultraviolet and blue coloration of males' throats are caused by a sophisticated multilayer system of iridophores and an underneath melanophore layer (Pérez i de Lanuza and Font 2014; Megía-Palma 2016) (Fig. 2a,c,e). Th white throat skin structure of females can be similar, but with disordered multilayer interference of guanine crystals in the iridophores (Fig. 2a,c) (Saenko et al. 2013). Dermis of the intensive yellow breast and belly of both sex can contain high concentration of lutein droplets in a xantophore layer with an iridophor layer which reflect yellow light; however, there is another peak in the ultraviolet spectral range (Martín and López 2009; Megía-Palma 2016) (Fig. 1b) Yellow color can depend on density and thickness of a xanthophore layer, and, furthermore the inside carotenoid pigment concentration; however, melanin density of melanophores can affect yellow coloration of the skin (Megía-Palma 2016). The dorsal green coloration of males and some females is likely to be produced by the two above process together (Fig. 2b,c,d). Most females have brown dorsal coloration which can be produced by thicker guanine crystals in ordered multilayer arrangement covered with some melanin pigments (Fig. 2f) (Saenko et al. 2013). Some lizards' brown skin structures also have some xanthophore to change the tone of the color (Fig. 2g) (Kuriyama et al. 2006).

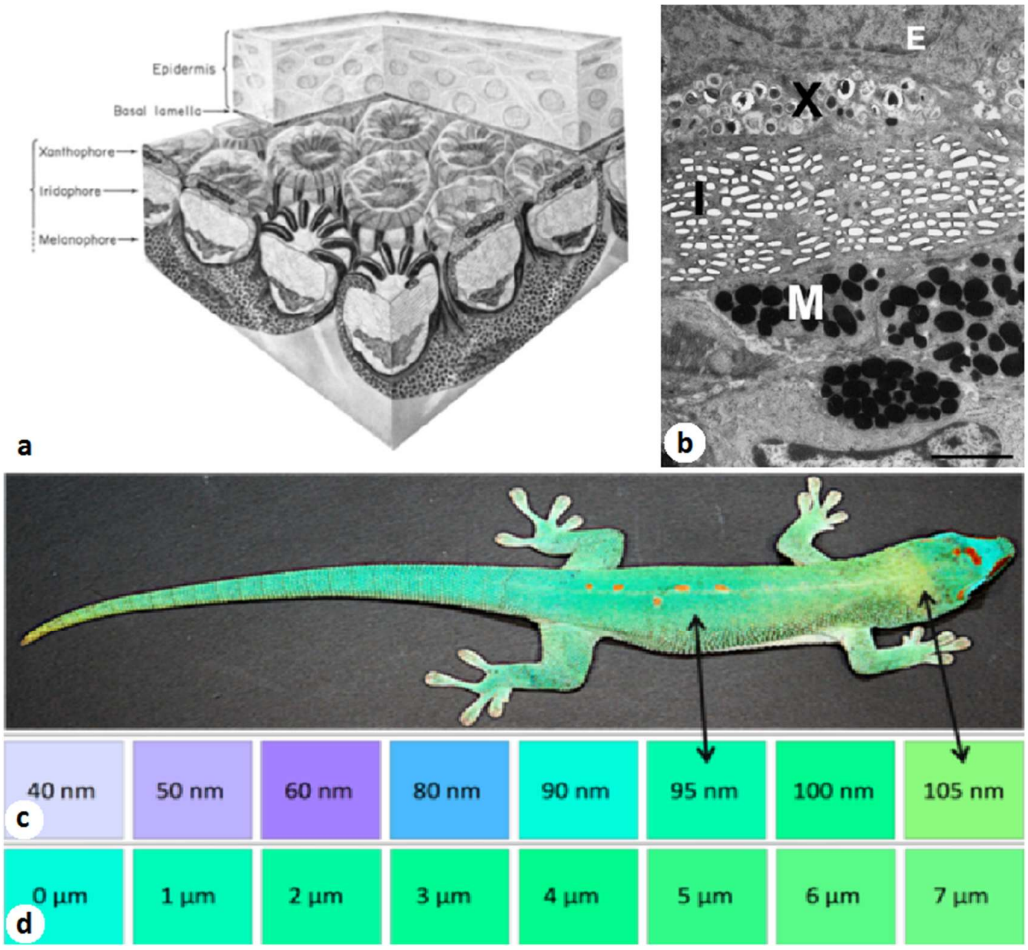

Fig. 1 (**a**) Schematic representation of a dermal chromatophore unit. (Figure reproduced from Bagnara et al. 1968 by Ligon and McCartney 2016). (**b**) Electron photomicrograph of dermal chromatophore unit in yellowish-white skin in *Plestiodon latiscutatus*. E, epidermal layer; X, xanthophores; I, iridophores; M, melanophores. Bar=2 µm (Kuriyama et al. 2006). (**c**) Color change by a 7 µm yellow pigment layer on top of a multilayer interference reflector with increasing spacing (40 to 105 nm) between layers of guanine crystals 80 nm thick. (**d**) Color change by varying thicknesses (0 to 7 µm) of a yellow pigment layer on top of a blue reflector in *Phelsuma grandis* (Saenko et al. 2013).

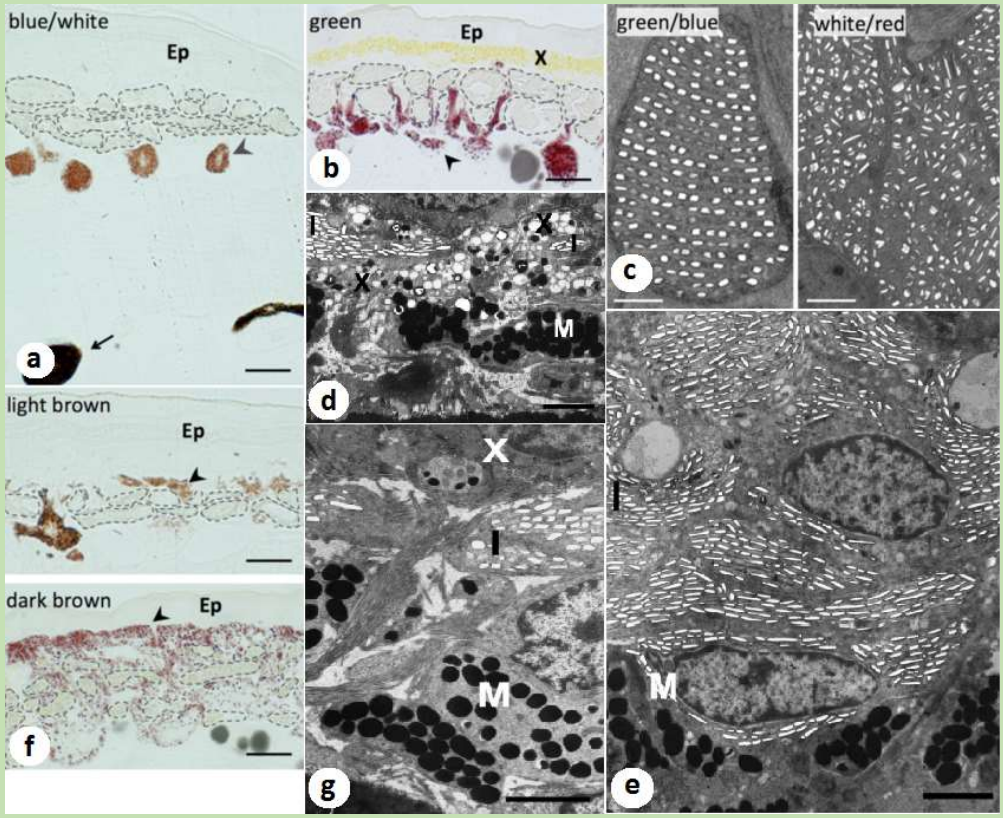

Fig. 2(a)(b)(f) Semi-thin cross-sections of skins of different colors in *Phelsuma* geckos: blue/white (**a**) green (**b**) light/dark brown (**f**) Arrows show melanophores, iridophores are outlined with dashed lines. Ep, epidermis; X, xanthophores. Bar = 10 µm (Saenko et al. 2013). **(d)(g)(e)** Electron photomicrograph of different structure of dermal chromatophore unit of green (**d**) brown (**g**) and blue (**e**) skin colorations in *Plestiodon latiscutatus* (Kuriyama et al. 2006)(**c**) Electron micrographs of iridophores in skin of blue/green and white colors in *Phelsuma* geckos. Bar = 1 µm.

Chemical communication

Beside visual signals, emission of chemical compounds (so-called semiochemicals) is a very widespread method of communication. A special subclass of semiochemicals are pheromones which are used in intraspecific communication (Law and Regnier 1971; Wyatt 2003). In invertebrates, usually, composition of pheromones is simple; they mostly consist of only a few compounds that attract the opposite sex. However, in vertebrates, chemical communication is more complicated by the existence of multicomponent chemical signals formed by very different kind of compounds that can deliver different multiple messages (Muller-Schwarze 2006). Chemical communication usually has important roles in social and sexual behavior of reptiles, thanks to their highly developed olfactory and vomeronasal

systems (Schwenk 1995; Halpern and Martínez-Marcos 2003; Martín and López 2014). Chemical signals are produced by large specialized holocrine glands (e.g. femoral, precloacal or cloacal glands) (Mason 1992; Labra et al. 2002). The relevance of chemical signals is indicated by the secretory activity regulation ability of reproductive hormones, such as testosterone, which favor production of more abundant secretion in males during the mating season (Fergusson et al. 1985; Mason 1992; Moore and Lindzey 1992; Alberts et al. 1992; Martins et al. 2006; Baeckens et al. 2016). Secretion consists of lipophilic and proteinaceous compounds. Proteins likely have a role in interspecific recognition because the intraspecific variability of proteinaceous components is low, although minor differences in proteinaceous compounds among individuals might be used in individual recognition (Alberts 1990, 1991; Alberts et al. 1993; Alberts and Werner 1993). Lipophilic compounds with higher interindividual variability may be more important for communication in a reproductive context (e.g. Martín and López 2006a; Kopena et al. 2011). Lipophilic components are more volatile and have a high degree of molecular diversity, thereby the potential information content of these chemical signals increases. The source of lipid components is the general metabolism and, thereby, the characteristics of chemical signals can be directly related with health and condition of the animal resulting in a reliable signal (Martín and López 2015). Typical lipophilic components in femoral or precloacal gland secretions of lizards are steroids and fatty acids, as major compounds, however minor compounds such as alcohols, esters of alcohols and carboxylic acids (=waxy esters), squalene, or tocopherols may also have important roles in communication (Weldon et al. 2008). Composition of femoral secretions can signal the age/size (López et al. 2003; Martín and López 2006a), morphological traits (López et al. 2006), parasite load, health state (Martín et al. 2007a; Martín et al. 2008), condition (Martín and López 2010a), attractiveness (Martín and López 2006b; Kopena et al. 2011), or dominance status (Martín et al. 2007b; Martín and López 2007) in different lizard species, confirming the relevant role of chemical signals in intra- and intersexual interactions of lizards.

Table 1 Lipophilic compounds found in femoral secretions of male *L. schreiberi* (López and Martín 2006). The relative amount of each component was determined as the percentage of the total ion current (TIC) and reported as the average (±1SE) for fifteen individuals. Characteristic ions (*m/z*) are reported for unidentified compounds.

RT[a] [min]	Compound	Mean ± SE	RT[a] [min]	Compound	Mean ± SE
25.3	Nonanoic acid	0.24 ± 0.06	51.8	Docosanoic acid	0.03 ± 0.02
27.9	Decanoic acid	0.28 ± 0.16	53.8	Unidentified??? (179, 291, 403)	0.02 ± 0.01
31.2	Dodecanol	0.22 ± 0.05			
32.9	Dodecanoic acid	1.34 ± 0.19	54.4	Tetracosanol	0.08 ± 0.02
35.9	Tetradecanol	1.22 ± 0.44	55.7	Squalene	0.20 ± 0.04
36.1	2-Pentadecanone	0.17 ± 0.04	56.8	Cholesta-3,5-diene	0.05 ± 0.01
37.4	Tetradecanoic acid	0.15 ± 0.03	50.2	Unidentified tocopherol derivative??? (179, 444)	0.46 ± 0.08
39.4	Pentadecanoic acid	0.06 ± 0.02			
40.1	Hexadecanol	0.08 ± 0.03	59.7	Cholesterol methyl ether	0.78 ± 0.08
40.3	2-Hexadecanone	0.05 ± 0.01	60.8	Cholesterol	6.43 ± 1.24
41.1	Hexadecenoic acid	0.48 ± 0.09	61.0	Cholestanol	12.30 ± 1.54
41.5	Hexadecanoic acid	3.43 ± 0.65	61.1	α-Tocopherol	18.26 ± 1.24
42.3	Heptadecanol	0.49 ± 0.08	61.7	Cholesta-3,5-dien-7-one	0.23 ± 0.03
43.3	Heptadecanoic acid	0.04 ± 0.01	61.9	Cholestan-3-one	0.63 ± 0.07
43.8	Octadecanol	0.34 ± 0.07	62.1	Ergost-22-en-3-ol	1.66 ± 0.09
44.2	4-Hydroxy-hexadecanoic acid γ-lactone	0.19 ± 0.03	63.2	Ergosta-7,22-dien-3-ol	0.31 ± 0.06
			63.5	Campesterol	3.90 ± 0.33
44.7	9,12-Octadecadienoic acid	0.56 ± 0.12	63.7	Ergostanol	16.54 ± 1.56
44.8	Octadecenoic acid	2.11 ± 0.29	64.2	4-Methyl-cholest-7-en-3-ol	0.22 ± 0.04
45.2	Octadecanoic acid	1.25 ± 0.23	64.9	γ-Ergostenol	1.42 ± 0.23
47.6	Eicosatetraenoic acid ethyl ester	0.07 ± 0.02	65.3	3,11-Dihydroxy-pregnan-20-one	0.15 ± 0.04
47.9	4-Hydroxy-octadecanoic acid methyl ester	0.10 ± 0.01	65.6	4,4-Dimethyl-cholest-8(14)-en-3-ol	0.10 ± 0.04
48.1	Eicosanol	0.03 ± 0.01	65.9	γ-Sitosterol	1.39 ± 0.21
48.3	Erucic acid	0.18 ± 0.06	66.3	Unidentified steroid (215, 233, 257, 383, 399, 416)	13.30 ± 2.20
48.6	Eicosanoic acid	0.87 ± 0.14			
50.3	Unidentified??? (113, 195, 307, 322)	0.09 ± 0.03	66.8	20-Methyl-pregn-20-en-3-ol	1.38 ± 0.23
			67.2	4,4-Dimethyl-cholesta-8,14-dien-3-ol	0.21 ± 0.06
51.3	4-Hydroxy-octadecanoic acid γ-lactone	0.16 ± 0.02			
			67.7	Stigmast-7-en-3-ol	5.74 ± 0.45

[a] RT, retention time.

Immune and oxidative costs of sexual signals

Honest signals require the existence of a link between the signal and some defining property of fitness. This link is usually supported by the cost of the signal. This cost can be manifested in several ways, such as production costs of color or chemical signals. Nevertheless, it requires internal processes of the organizations that are able to influence the expression of signals by extent of the production cost, evolving trade-offs between the sexual signal system and essential organismal systems. The secondary sexual characteristics usually have a role as sexual signals and their expressions are strongly dependent on the hormonal system. In the breeding season, androgens, such as testosterone, level is increased and supports the development of sexual signals (e.g. Silverin 1980; Regan 1981; Fox 1983). Nevertheless, the hormonal system may affect the immune system, and, thus, it can change the defensive capability of the body. Testosterone has immunosuppressive effects (Bradley 1987; Pickering and Christie 1980). These two important effects of testosterone are linked

in the immunocompetence-handicap hypothesis (Folstad and Karter 1992). In house sparrows, testosterone increases size of the melanin-based bibs, but has negative effects on the immune responses, although corticosterone is likely mediated in these processes (Evans et al. 2000; Buchanan et al. 2003). Testosterone is also able to increase the amount of secretion of chemical signals (Fergusson et al. 1985; Alberts et al. 1992, Baeckens et al. 2016), but it may alter the chemical composition of the signal that is preferred by females (Martín et al. 2007c). However, materials of signals are also able to function in immunological processes. Melanin production and immunity are linked by genetical, biochemical and functional pathways (Mackintosh 2001). In invertebrates, melanin is one of the basic material against microbes (Sugumaran 2002), while in vertebrates, except mammals, links of melanin and immunity are less known, however melanin may participate in some relevant process of defense (reviewed in Mackintosh 2001). By its property of tissue strengthing, melanin supports the skin as the first line of the defense system (e.g. Bonser 1995). Genetical link is demonstrated by several diseases which cause both albinism and impaired immunity (Introne et al. 1999; Baumeister et al. 2000; Spritz 2000) or by the protein called attractin which appears to regulate melanization and immunity (Duke-Cohan et al. 1998; Duke-Cohan et al. 2002). The melanin production regulator α-melanocyte stimulating hormone (α-MSH) also itself regulates the immune system, has antipyretic, anti-inflammation and antimicrobial effects, decreases lipopolysaccharide (LPS) induced nitric-oxide (NO) synthesis, and activates phagocytic cells (Kalden et al. 1999; Harris and Bird 2000; Tsatmali et al. 2000; Catania et al. 2000; Ichiyama et al. 2000). Melanin can play a role in regulation of cytokine activity. Synthetic melanin is able to suppress tumor necrosis factors and inhibit interleukins in response to LPS to decrease inflammation (Mohagheghpour et al. 2000). For effect of LPS, melanocytes produce NO and human melanocyte increases melanin synthesis as a reaction to increased NO levels (Tsatmali et al. 2000; Sasaki et al. 2000). Melanin production also increases in case of histamine releasing, which is the first reaction of the body to acute trauma or diseases (Yoshida et al. 2000). The melanin producer melanocytes and inside melanosomes also show phagocytical and lisosomal enzyme activity in antigen processes, probably these are components of the skin

immune system (Le Poole et al. 1993b). Furthermore, melanocytes can have a role as antigen presenting cells that can elicit T-cell proliferation (Le Poole et al. 1993a,b) and be able to produce interleukins (Smit et al. 1993). Carotenoids itself also have immunostimulant effects. Carotenoids are able to increase antibody production, limphocyte proliferation, number of macrophags, phagocytosis, and disease resistance in birds and mammals (reviewed in Surai 2002), and, furthermore, carotenoids can influence expression of immune-related genes (Geissmann et al. 2003). Vitamin E, which is an important component of lizards' chemical signals, is also a relevant component of the immune system. Vitamin E increases antibody production, macrophag and complement activity, limphocyte proliferation, recovery and survival rate of vertebrates (Surai 2002). The immune system has special relationships with the antioxidant system. Most of free radicals are produced by oxidative burst in mitochondries (Barja 2007), but other redox reactions also produce free radicals, such as macrophags and neutrophils that release ROS, RNS which react fast and effectively to patogens, but in this process the host cells also can be hurtled or killed by oxidative degradation (Nathan and Shiloh 2000; Coleman 2001). Immune cells are rich in polyunsaturated fatty acids, which are very susceptible to attacks of free radicals. Carotenoids and vitamin E increase membrane resistance against lysis activated by the innate immune system (e.g. Chew and Park 2004; Hong et al. 2004), even the smallest lipid peroxidation can change membrane structure and properties, inhibiting the antigen recognition, receptor expression, secretion of antibodies and cytokines, etc., thereby damaging the processes of the immune system (Wu and Meydani 1998). Melanins are valuable free-radical scavengers in several organism such as fungi (Shcherba et al. 2000), amphibians (Geremia et al. 1984) and mammals (Różanowska et al. 1999; Kasraee et al. 2003). However, melanin production can also depend on the level of the most important intracellular antioxidant (Anderson 1998; Wu et al. 2004). When glutathione level is high, the melanin production is inhibited, but when glutathione level is low, melanin production is started in melanocytes. But, with decreasing glutathione levels, the body requires another effective antioxidant to substitute the antioxidant effect of glutathione in the cells, otherwise, the lack of alternative antioxidant sources would infer very high oxidative stress (Halprin

and Ohkawara 1966; Benedetto et al. 1981; Galván and Alonso-Alvarez 2008). Vitamin E is one the most useful fat-soluble antioxidants in the wild life (e.g Di Mascio et al. 1991). These antioxidants, which have important roles in developing of sexual signals in many species, can influence mate preference through supporting the immune system or only as antioxidants, signaling good fitness properties of the bearer (von Schantz et al. 1999). Carotenoids are also antioxidants *in vitro* (Krinsky and Yeum 2003), however their *in vivo* role is controversial. Carotenoids may affect the immune and/or antioxidant systems (Lozano 1994; von Schantz et al. 1999; Møller et al. 2000). However, carotenoids transform toxic aldehids and lose their color by reaction with free radicals (Palozza 1998), while antioxidants without color (e.g. vitamin E or C) are more effective radical scavengers and vitamin C can support to vitamin E to transform back to the original state. Thus, maybe carotenoids have other more important roles in the body (sexual signal, immunity, etc.) than to be an antioxidant, and maybe they are only simple sufferers from free radicals if the antioxidant system is not adequate. So maybe carotenoid-based sexual signals just indicate the state of the true antioxidant system (Hartley and Kennedy 2004).

Signaling of female quality and mother investment

In spite of male sexually selected signals are very well-researched in behavioral ecology, signals of females are less known. But females usually have elaborated ornaments which sometimes monomorph, sometimes only similar and partly matched with male signals, but in some cases females possess completely independent ornaments from the other sex. According to a previous theory, female ornaments are only a result of non-adaptive process of intersexual genetic correlation due to the sexual or natural selectional effect on males' genome which are inherited by females as well (Lande 1980). In this case, conspicuous ornaments have negative effects on female survival possibility and fecundity, especially in case of maternal care due to higher predation risk. Common yellowthroat (*Geothlypis trichas*) females with more vivid yellow bibs suffered more likely total brood loss due to predation (Freeman-Gallant et al. 2014).

Female ornaments can also evolve by direct selection. Females can compete for mates, especially in case of operational sex ratio (OSR) biased species which can result in reversed sex-roles, such as in pipefish (*Syngnathus typhle*) males that prefer striped-patterned females which ornaments can indicate competitiveness of females for males (Berglund and Rosenqvist 2001). Females use signals to indicate competition capability for resources. For example, in dichromatic Eclectus parrots (*Eclectus roratus*) where females are bright red and blue while males are bright green, females have very aggressive encounters for the rare breeding hollows (Heinsohn et al. 2005). Female ornaments can indicate female quality informing about their own health state and condition (Johnsen et al. 1996; Roulin et al. 2001; Roulin et al. 2008; Dreiss and Roulin 2010; Kelly et al. 2012). Pied flycatcher (*Ficedula hypoleuca*) females with forehead patches have less parasites than females without forehead patches (Potti and Merino 1996). Brighter red Arctic charr (*Salvelinus alpinus*) females have less parasites than drabber females (Skarstein and Folstad 1996). Furthermore, these signals can show the possible mother fecundity and investment into her offspring (Jawor et al. 2004; Siefferman and Hill 2005; Roulin et al. 2010; Potti et al. 2013; Remeš and Matysioková 2013). However, although genetic correlation and direct selection give opposite predictions on the relationship between female signals and female fitness, they are not mutually exclusive phenomena. Vestigial traits from genetic correlation can be a base to evolve other ornaments by sexual selection. Furthermore, cooperation of these two processes may occur, an attractive female partner can result benefitial for a male by producing not only showy daughters but also more attractive sons and *vice versa* (Amundsen 2000).

Evolution of mate choice can be influenced by the species' breeding system, operational sex ratio (OSR), parental investments, cost of pair searching and the variance of quality among females. In case of biased OSR, the rare sex is choosy, thus female biased OSR can result in reversed sex roles and elaborated ornaments. But at balanced OSR, when females mate more males and "sperm mixing" exists, a higher quantity of sperm result in a higher fertilizing success for males in sperm competition (Parker 1982; Parker et al. 1996, 1997). Usually males ejaculate much more sperm than necessary to fertilize of all eggs.

However, production of a single sperm is cheap (Dewsbury 1982), but in polygynous or promiscuous mating systems, the increased requirement of sperm transfer can be costly by limited ejaculate volume (Squires et al. 1978; Pitnick and Markow 1994; Cook and Gage 1995; Savalli and Fox 1999). Male fowls (*Gallus gallus*) invest more sperm into more ornamented females, especially the good quality males (Cornwallis and Birkhead 2007). Theoretically, evolution of male mating preference is favored and males have direct benefits if females' quality have high variance, or mate search cost is low, or males' parental investment is high (Burley 1977; Parker 1983; Owens and Thompson 1994; Johnstone et al. 1996; Kokko and Monaghan 2001). In most of polygynous species where females have ornaments, females access to more males and have direct benefits from multiple pair mating (Clutton-Brock 2009). When mating rate and quality variance is similar in the two sex, or both sex invest in parental care, mutual choosiness is expected (Burley 1977; Owens and Thompson 1994; Johnstone et al. 1996). As higher the parental investment of a sex is, as choosier it is, and lower quality individuals discriminate less than good quality ones (Burley 1977). However, there are some proofs that the real reason for choosiness is not parental care *per se*, but rather the "time-out" of sexual competition influencing potential reproductive rate. Thus, other time-consuming behavioral investments, such as e.g. mate guarding, can have similar effects on choosiness (Parker and Simmons 1996).

Honesty of female signals functions through the existence of a cost, as in case of males. However, this cost can cause a trade-off between ornaments and fecundity. Thus, sexual selection on female ornaments can be self-limited. If males choose females by their direct fecundity benefits, they avoid females with too elaborated signals for the cost of fecundity, but if females have to invest into ornaments anyway, males will be not able to estimate potential fecundity of females. Thereby,

stabilization selection affects female sexual signals (Fitzpatrick et al. 1995). Carotenoid-based pelvic spine coloration of female Three-spined sticklebacks (*Gasterosteus aculeatus*) negatively relate with carotenoid content of their eggs and males do not prefer females with redder spine (Nordeide 2002; Nordeide et al. 2006). Nevertheless, female traits can signal directly their investment into offspring, without costs. In certain species, body size or body condition can predict number or size of offspring (Itzkowitz et al. 1998; Bonduriansky 2001). Females can influence quality of their offspring by genetical and non-genetical mechanisms. Females allocate nutrients and other important biochemicals into the embryos through the placenta or into egg yolk. Egg yolk mainly consist of proteins and lipids as nutrients (Thompson and Speake 2002), plus fatty acids, antibodies, steroid hormones and antioxidants (Schwabl 1996; Wiegand 1996; Speake et al. 2001; Gasparini et al. 2001; Izquierdo et al. 2001; Boulinier and Staszewski 2008). Rapid embryonic growth rate due to mitosis and respiration results in high oxidative levels inside the eggs (Blount et al. 2000; Surai et al. 2001a). Thus, antioxidant content of eggs is a very important aspect of embryonic development, neonatal growth and survival of the offspring (George et al. 2001; Izquierdo et al. 2001; Johnston et al. 2007). Conspicuous color signals, such as carotenoid or pteridin-based ornaments can signal carotenoid/pteridin content of the body, which are important antioxidants, or can reflect the other antioxidants supply of the body which allow to maintenance the colour signals. Size or intensity of these color patches can be related with antioxidant content of the eggs. Lesser black-backed gulls (*Larus fuscus*) and Zebra finches (*Taeniopygia guttata*) females fed a high-carotenoid diet invest more to color intensity of ornament and allocate more antioxidants into eggs (Blount et al. 2002; McGraw et al. 2005). Striped plateau lizards (*Sceloporus virgatus*) females have pteridin-based orange throat patches which reach peak of color intensity at around ovulation of females and are preferred by males. Size of throat patches positively relate with body condition and negatively with parasite load of females, while orange chroma positively relate with body size, thus indicating phenotypic and genetical quality of the females. Furthermore, patch size positively correlates with

antioxidant content and average mass of the eggs, and thereby, predicts better condition and higher running speed of the offspring (Weiss 2006; Weiss et al. 2009; Weiss et al. 2011). Structural coloration can also predict female fitness; Blue tits (*Cyanistes caeruleus*) females have ultraviolet crown, as males, which predict egg size and numbers of fledgings (Szigeti et al. 2007; Henderson et al. 2013).

Predation risk as a cost of conspicuous signals and mate search activity

Predation is the one of the most important selection pressures that determines the form (Endler 1991) and behavior of animals (Lima 1998). Since sexual signals and mate search activity can increase future fitness, conspicuousness is increased not only for conspecifics but also for predators (Møller 1989; Magnhagen 1991; Forsman and Shine 1995) eliciting higher predation risk (Stuart-Fox et al. 2003; Husak et al. 2006). Increased predation risk can cause biased predation to the most conspicuous gender, which usually results in significant male bias in capture of predators (Cade 1960; Lindberg 1983; Møller et al. 2011). Prey plumage brightness is the most important factor determining vulnerability to predation by Sparrowhawks (*Accipiter nisus*) (Huhta et al. 2003). Predation of Western Green lizards (*Lacerta bilineata*) - a close relative lizard to *L. schreiberi* that has similar sexual signals - by Eurasian kestrel is male-biased (Costantini et al. 2007). Animals can adapt to predation risk by decreasing the signal "intensity", such as in Trinidadian guppies (*Poecilia reticulate*), where reduced risk of predation elicits an increase in conspicuousness of their signals (Endler 1980; Endler 1984).

To compensate this higher risk, signaling animals can also modify their behavior (Lima and Dill 1990; Magnhagen 1991). Level of chemical alarm cues in water elicits decreased activity in male red-spotted newts (*Notophthalmus viridescens*) (Rohr and Madison 2001). Conspicuousness of male signals usually correlates positively with shyness (Forsman and Appelqvist 1998; Martín and López 1999b; Hedrick 2000; Cuadrado et al. 2001; Lindström et al. 2006; but see Godin and Dugatkin 1996). Males of *Iberolacerta monticola* lizards with more blue lateral ocelli behave shier, and spend more time in the

refuge after simulated predator attacks than males with less elaborated signals (Cabido et al. 2009). Behavior compensation in sexually dichromatic species can be manifested in that the most ornamented gender allows predator to approach less close than the other gender with better cryptic properties does (Lailvaux et al. 2003; Martín and López 1999a; Smith 1996, 1997; Cooper and Wilson 2007; Whiting 2002). However, testosterone can decrease approach distance (Tryjanowski and Møller unpublished data in Cooper and Blumstein 2015). Male green lizards, *Lacerta viridis*, have longer flight initiation distances than females before and after the mating season, but during the mating season the difference dissipates (Majláth and Majláthová 2009). Approach distances also vary among species, being inversely correlated with the degree of cryptic coloration (Heatwole 1968; Johnson 1970), or, within a given species, as a function of the degree of conspicuousness in different microhabitats (Cooper 1998). Many animals are less responsive to predators during the reproductive seasons (Brown and Shine 2004). Outside of the mating season, without mate search activity and with decreased signal conspicuousness, males can change their antipredator behavior. Certain males of darter (*Etheostoma flabellare*), which change dramatically their breeding coloration to intense and contrasting orange, blue, yellow and red colors on their sides, fins and throats, usually use fleeing tactics, while in the non-breeding season their color change to dull and they alter their escape strategy to cryptic ”freezing” in response to a predator threat (Radabaugh 1989). Male Iberian rock lizards have longer hiding times in the non-breeding than in the breeding season (Martín et al. 2003a). Thus, adaptation to actual predation risk and shifts of antipredator behavior may be able to compensate (at least until a certain level) the natural selection cost of elaborated conspicuous signals.

Main Objectives

The main goal of this thesis is to study the function, evolution and maintenance of the multiple sexual signals that *L. schreiberi* lizards use in sexual selection processes and the effects of natural selection pressure on these signals on antipredator strategies of lizards. The next six chapters will elaborate this through the following concrete objectives:

Objective I. Conspicuous sexual signals, mate searching and mate guarding can greatly enhance the predation risk on males of sexually dichromatic species. In this study we examined whether the intersexual differences in coloration and the breeding constrains affect preventive risk-taking behavior (i.e., the overall distance to the nearest potential refuge that the lizard maintains before an attack) and the active escape decisions in an emergency situation when an attack occurs (i.e., the distance to the predator when a lizard starts fleeing) or whether only the current environmental conditions influence the antipredatory behavior of this lizard against a predator. **(Chapter I)**

Objective II. Costs of carotenoid-based sexual signals are well-researched in birds, but very unknown in lower vertebrates. Developmental and maintenance costs of other visual and chemical signals are less investigated in all the animal kingdom. In the next three studies, we investigated the relationship of the antioxidant and immune systems with highly elaborated multiple sexual signals of male *L. schreiberi*, which include both carotenoid, melanin and structural coloration and chemical signals.

A, This study examined whether supplementary antioxidants in the diet, such as pigmentary carotenoids and non-pigmentary vitamin E, influence different visual and chemical sexual signals. **(Chapter II)**

B, This study examined the effects of dietary carotenoids and vitamin E on body condition and PHA-induced immune response. **(Chapter III)**

C, In this study we investigated whether the costs of an immune activation affected the expression of sexual signals of different types (visual and chemical), and the interactive effects with a dietary supplementation of an important antioxidant, vitamin E. **(Chapter IV)**

Objective III. Movement patterns of animals can reveal social relationships and mating behavior of the species. In this study, we explored home range sizes and intra- and interspecific overlapping of home ranges related with sexual signals of both male and female lizards to analyze how dominance of males and attractiveness of both genders may influence their space use. **(Chapter V)**

Objective IV. Female sexual signals are less researched comparing to males' ones. We compared color characteristics of both gender and, furthermore, we examined whether carotenoid, melanin, or structural-based coloration of *L. schreiberi* females have a signaling function of any health parameter or components of reproductive potential of these females. **(Chapter VI)**

Methods

The Iberian green lizard (*Lacerta schreiberi*)

The Iberian or Schreiber's green lizard is an endemic species in the Iberian Peninsula. It is mainly distributed in the Atlantic climated northwest and the Mediterranean mountainous central part of the peninsula and a few southern geographically isolated populations where at least 800 mm precipitation falls per year (Brito et al. 1996; Marco and Pollo 1993). It occurs from sea level up to 2100 meters but it prefers mountainous regions. This species mainly inhabits humid places, such as margins of streams, but also can be found in areas with rocks and bushes (Brito et al. 1999).

Fig. 3 Distribution of *L. schreiberi*. Yellow color shows resident, red color shows possibly extinct populations (Sá-Sousa et al. 2009). The red arrow shows the location of the research area, Valle de La Fuenfría and Valle de Navalmedio.

Our study area was located in two contiguous small valleys ('Valle de La Fuenfría' and 'Valle de Navalmedio') (40°44' N, 4°02' W; Cercedilla, Madrid Province, Spain) in the

Guadarrama mountains, from 1300 m to 1600 m, mostly on margins of streams. The dominant vegetation consists of *Pinus sylvestris* forest, with shrubs such as *Juniperus communis*, *Rosa canina*, *Rubus ulmifolius* and *Cytisus scoparius*.

Fig. 4 Typical habitat of *L. schreiberi* in Valle de La Fuenfria

Usually, *L. schreiberi* is active from March to October, however there can be even 50 days of difference between spring emergence of sexes in the Central Mountain System of Spain (Marco 1994). The mating season starts at the end of April and lasts until the end of May, egg laying occurs from the end of May to the end of June. It has only one clutch per year with 7-24 eggs, except in Asturias, where the good climatological conditions can allow two clutches per year (Braña 1982), however those have fewer eggs than in the areas where only single clutches are produced. After 65-110 days of incubation time, the first hatchlings appear in the second half of August and show high daily activity until the first frost of October. The Iberian green lizard is a diurnal species with high daily activity in spring, because of the mating season, but this activity shows a drastic decrease in summer, particularly in the case of males. In September activity increases a bit, but in October, with the first frost lizards begin the winter hibernation.

L. schreiberi spend in movement only 10.75 % of its activity time, therefore it is likely that this species uses both sit-and-wait and active foraging strategies (Verwaijen and Van Damme 2008, personal observation). Their diet is very variable; it is mainly based on arthropods such as *Coleoptera, Formicidae, Diptera, Arachnidae, Orthoptera* and *Homoptera* (López 1982; Pérez-Mellado 1983; Llorente and Pérez-Mellado 1988; Domínguez and Salvador 1990; Ortega-Rubio 1991), but very rarely also feed small lizards and nestlings of small song birds, and, furthermore, some vegetables and fruits of *Rosaceae*

(Llorente and Pérez-Mellado 1988). This species shows strong sexual dimorphism and dichromatism. Males and females of the same age do not show differences in body length (Marco 1995), but males have bigger heads and more collar scales than females, and females have longer and larger abdomens and more transversal rows of ventral scales (Galán 1984; Marco 1994; Braña 1996). Males have, especially during the breeding season, green dorsal coloration with small black spots, yellow chest and ventral coloration, and bright blue and UV throat and mental coloration (Martín and López 2009). In contrast, females have brown or green dorsal coloration, with yellow chest and belly and whitish throat.

Fig. 5 A male (left) and different color variations of females (center and right) of *L. schreiberi.*

Interindividual variation in the characteristics of coloration of males can be related to variation in morphology, health state, dominance and pairing status, but different relationships, probably based on different physiological trade-offs, are found for each color signal (Martín and López 2009). On the other hand, male *L. schreiberi* has well developed femoral glands in the hindlimbs that produce abundant secretions during the mating season. Secretions contain many lipophilic compounds, including several steroids, fatty acids, alcohols and, interestingly, large amounts of α-tocopherol (= vitamin E) (López and Martín 2006)(Table 1). In the closely related European green lizard (*L. viridis*), which has very similar compounds in secretions (Kopena et al. 2009), females preferred to use areas scent marked by males with experimentally increased vitamin E levels in their secretions (Kopena et al. 2011), suggesting that the possible cost of allocating antioxidant vitamin E to secretions may confer reliability to chemical signals of green lizards. The breeding system of *L.*

schreiberi is polygynandry (Marco and Pérez-Mellado 1999). Space using behavior is sedentary (Marco 1996), home ranges of males and females overlap with each other (Marco and Pérez-Mellado 1999). According to earlier studies, this species is non-territorial, however it is proven they defend mates (Marco and Pérez-Mellado 1999; Martín and López 2009), but they also defend basking places and refuges from other males (personal observation). Larger males have more and larger mates and, furthermore, a higher mating success, but these do not relate with home range size (Marco and Pérez-Mellado 1999). More dominant males have higher heads, bluer, brighter throats, and more saturated green back coloration (Martín and López 2009). During intrasexual interactions, males show many types of displays and behaviors, such as biting and wrestling, in case of a direct fight between two similar sized animals, chasing, if they have a bigger size difference, or threaten by chest upraising and displaying throat coloration (Marco and Pérez-Mellado 1999, personal observation). Also, males likely use chemicals in femoral secretions to sign the owner quality to intruders, as related species do (López et al. 1998; Aragón et al. 2000, 2001).

The back coloration and pattern of Iberian green lizards ensure good camouflage in vegetation, and sometimes lizards permit to approach the predators for some centimeters in cold days. However, lizards are very conspicuous in open rocky environments, particularly in the mating season. Lizards prefer refuge rich areas, such as rocks, holes in the ground, dense bushes, but in case of a predator attack lizards can climb high to tree trunks or jump into water of a stream where they can wait for some minutes under water (García-París et al. 1989; Marco 1994). However, refuge use has costs in terms of body temperature loosing, with temperatures outside and inside a refuge having opposite effects on refuge use decisions (Martín and López 2010b). The higher the temperature is outside the refuge and, therefore, the higher the body temperature of the lizard is, the longer the time that the lizard can stay inside a refuge before reaching a 'critical' low body temperature. But in case of refuge temperature, the colder is inside. the faster the lizard reaches a 'critical' low body temperature and the longer the time it has to stay in the refuge. Because of increasing of emerging risk by lower escape performance, lizards would need to compensate by remaining in a refuge for longer to scan the surroundings before emerging to ensure that the risk of a

new attack decreased. Therefore, when deciding refuge use, *L. schreiberi* lizards seem to consider physiological costs of being at low temperatures and also the risk of emerging with low escape performance (Martín and López 2010b).

On his distribution area, *L. schreiberi* have many predators; mammals such as European pine martens (*Martes martes*), European otters (*Lutra lutra*), common genets (*Genetta genetta*), birds such as Montagu's harriers (*Circus pygargus*), white storks (*Ciconia ciconia*), European honey buzzards (*Pernis apivorus*), tawny owls (*Strix aluco*), and reptiles such as smooth snakes (*Coronella austriaca*) (Callejo et al. 1979; Alegre et al. 1989; Martín and López 1990; Clevenger 1993; Galán and Fernández-Arias, 1993; Marco 1994; Garcia-Dios 2006). In our study area, we observed as potential predators: common kestrels (*Falco tinnunculus*), common buzzards (*Buteo buteo*), booted eagles (*Hieraaetus pennatus*), smooth snakes (*Coronella austriaca*), grass snakes (*Natrix natrix*), domesticated cats (*Felis catus*), dogs (*Canis familiaris*) and foxes (*Vulpes vulpes*) (personal observation).

L. schreiberi is included in the IUCN list, as a "near threatened" species and it is included in the Red Book of Spanish Vertebrates ("Libro Rojo de los Vertebrados de España") as in "endangered" status. Furthermore, in the II Annex of the National Catalogue of Protected Species appears as of "special interest". The main dangers for conservation of this species are human activity in their habitat, such as water contamination, bank destruction, altering of stream beds, water extraction of streams and massive reforestation (Brito et al. 1999). The global warming also has negative effects on humid habitats and therefore on their flora and fauna. This process will decrease the suitable habitats and populations will be isolated. With increase of fragmentation, in some cases *L. schreiberi* can suffer bottle-neck effects or in other cases populations of this species can become extinct. Climate change can decrease the distribution area of *L. schreiberi*; while in the northwest this might have less impact, populations in central and east part of mountains of the Iberian Central System and the southern isolated populations probably might become extinct for 2080 (Rödder and Schulte 2010).

Field Study

Escape Behavior

We searched for lizards between 10.00 and 18.00 h. The observations were carried out in sunny, warm and unwindy weather. The same observer (RK wearing the same clothing simulated an approaching predator. This is a conventional method in studies of lizard escape behavior, because lizards identify humans as natural predators (e.g. Braña 1993; Bulova 1994; Martin and López 1995; Martín and López 1999b; Martín and López 2000 a,b; Martín et al. 2003b; Cooper 1997a,b,c; Amo et al. 2003, 2005). The observer walked at approx. 1 m/s until an adult lizard was located. After this, the observer simulated a predatory attack by approaching the lizard directly at a slow speed (approx. 0.6 m/s) until the lizard fled. We recorded the sex of animals (sexual dichromatism was easily discerned by sight) and the following behavioral variables: (i) 'refuge distance', the distance between the lizard and the refuge it later used to hide, (ii) the flight initiation distance ('FID'), the distance between the observer and the lizard when the lizard started fleeing and (iii) 'escape angle', which was the angle formed by the line connecting the initial position of the lizard and the observer and the line connecting the lizard and the refuge or first stopping point after escaping (Martín and López 1996; Cooper 1997b). An escape angle of 0° indicated the escape direction was directly away from the predator, while 180° meant the lizards escaped directly towards the approaching predator (Martín and López 1996). The orientation of escape direction was not considered because it was immaterial whether movements were to right or left, as it presumably does not affect the probability of escape (Cooper 1997a). We also noted the microhabitat in which the lizard was initially located (grass vs. open rocks). The observations were made in different parts of a natural large field area where lizard density was high. Therefore, we assumed that the chance of repeated observations of the same individuals was low, and considered the data points to be independent (Bulova 1994; Martín and López 1995; Cooper 1997c).

Home Range Analyses

We individually marked captured lizards by using long lasting nail polish, with a combination of three small color marks placed on three body locations (head, back, base of the tail). After the observations, animals lose these marks naturally and nail polish did not cause any health damage for them. We released animals exactly at the same place where we had collected them one or two days before. This area is more or less closed for the animals because the surrounding pine forest was a suboptimal habitat (i.e. it had less light, less refuges and probably less food), and the closest optimal open area was at least 500m, where we never collected animals from. Furthermore, we had never seen adult lizards in the patch surrounding the pine forest (five years long personal observation). The observation period lasted for 30 days, when the weather let the animals to be active. Every day from 900 h to 1900 h, I checked the localization of sighted marked lizards every hour by a binocular, identifying individuals by their color code. The observation area was patchy with shrubs, rocks and weeds, where the detectability can differ, thus we compensated this difference by spent time to reach similar probabilities of detection in all areas. The location of lizards was determined with a GPS (Garmin) using a fixed transect in every hour. To ensure independence of data points, we collected only one data point per hour per animal.

Captivity experiments

Lizard capture and husbandry

Lizards were captured by using a fishing line noose that was tied to a fishing rod. We immediately transferred lizards to "El Ventorrillo" field station. During all the experiments, lizards were individually kept in outdoor 51×36×28 cm PVC terraria containing coconut fiber substratum and rocks for cover. PVC terraria were covered with a metallic net to protect them against predators. Terraria were located on a half-shady area, similar to the original habitat of lizards. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*), house crickets (*Acheta domesticus*), and common black crickets (*Gryllus*

assimilis) dusted with calcium powder (and vitamin powder if the experiment design allowed it), and water was provided *ad libitum*. At the end of the study, all lizards were released at their exact capture sites in good condition.

Fig. 6 Outdoor husbandry.

Morphological measurements of lizards

Basic measurements:

After capturing males I measured immediately the snout-to-vent-length (SVL), tail length (from the cloaca), head length, head height, and head width with a digital caliper (Mitutoyo) to the nearest 0.01mm. Body weight was measured with a digital balance to the nearest 0.1 g. The number of femoral pores was counted from photographs.

Derived variables:

"Body condition" was calculated as the residuals from the regression equation of ln mass (in grams) on ln SVL (in millimeters), which may represent an index of the relative amount of fat stored and hence an estimation of individual physical condition or nutritional status (Bonnet and Naulleau 1994). Only males with complete or entirely regenerated tails were considered to calculate body condition.

Coloration measurements

We measured reflectance of lizards' coloration from 300 to 700 nm using an Ocean Optics USB2000 spectroradiometer with a DT-1000-MINI Deuterium–Halogen light source (Ocean Optics, Inc., Dunedin, FL, USA). This range of wavelengths coincides with the

spectral sensitivity measured for other lizard species, which includes visual perception in the UV range (Fleishman et al. 1993; Loew et al. 2002). To exclude ambient light and standardize measuring distance, a cylindrical metallic tube was mounted on the bifurcated fiber optic probe (Montgomerie 2006). The probe was held at a 90° angle to the skin, and reflectance was always measured by the same person (PL). We measured coloration of three visually distinct large patches of coloration ("blue" throat, "yellow" chest, and "green" dorsum) at three standardized spots: the middle of the throat (between the last chin shields and the collar; "throat"), the chest (just anterior to the two forelimbs at the middle of the second row of scales prior to the collar; "chest"), and the dorsum (in the middle point above the two forelimbs; "dorsal").

We mathematically summarized the spectra using principal component analyses (PCAs) (Endler 1990; Cuthill et al. 1999; Grill and Rush 2000). The PCA summarizes all of the information about the shape of complex reflectance spectra, including bimodal ones like those found in *L. schreiberi* (see Martín and López 2009) into a few independent PCs (Montgomerie 2006). In PCA of spectral data, PC1 represents variation in intensity of coloration or brightness, and subsequent PCs represent combinations of hue and chroma (Endler 1990; Cuthill et al. 1999; Grill and Rush 2000). Also, the PCA identifies those sections of the spectrum (wavelength regions) that are contributing to the observed variation, independently of their "importance" in terms of contribution to the total amount of reflectance (Montgomerie 2006). Principal component analyses were performed separately for each part of the body (throat, chest and dorsal) including spectra for all males.

In addition, to clarify the role of carotenoids in coloration, from the raw spectral reflectance data of each body part, we calculated the carotenoid chroma $[(R_{700nm}-R_{450nm})/R_{700nm}]$, which represents relative reflectance around peak absorbance of carotenoids, thus indicating levels of carotenoid pigments incorporated into the integument (Johnsen et al. 2003; Peters et al. 2004). We subsequently correlated carotenoid chroma with the PC scores to explore whether variation in coloration defined by these PC scores could be determined by carotenoid levels.

Chemical analyses of femoral secretion

We collected femoral secretion of males directly into glass vials with glass inserts, later closed with Teflon-lined stoppers and stored at -20ºC. Samples were analyzed by gas chromatography-mass spectrometry (Finnigan-ThermoQuest GC-MS Trace 2000, Thermo-Quest Corporation, Austin, Texas) equipped with a Supelco-Equity-5 (Bellefonte, PA) column temperature programmed (50–280 ºC at 5 ºC/min and 280 ºC for 30 min). 2µl of each sample dissolved in 250µl of n-hexane (Sigma, capillary GC grade), were injected splitless mode with an inlet temperature of 250 ºC. The carrier gas was helium at 30cm/s. Ionization by electron impact (70 eV) was carried out at 280 ºC. Mass spectral fragments below m/z=39 were not recorded. Impurities identified in the solvent and/ or the control samples were not reported. Compounds were identified by comparison of mass spectra in the NIST/EPA/NIH (NIST 02, www.nist.gov) library, and later confirmed with authentic standards when these were available. The relative amount of each compound was determined as the percent of the total ion current (TIC) area transformed following Aitchison's formula: [Zij=ln(Yij/g(Yj)], where Zij is the standardized peak area i for individual j, Yij is the peak area i for individual j, and $g(Yj)$ is the geometric mean of all peaks for individual j (Aitchison 1986).

Immune response measurement and immune activation

One of the most widespread methods for measuring *in vivo* the immune response is the phytohaemagglutinin (PHA) skin-swelling test (Smits et al. 1999; Kennedy and Nager 2006; de Bellocq et al. 2007; Ardia 2008). PHA is a plant lectin that induces an artificial activation of the immune system. Although, this test was first referred as an indicator of T-cell-mediated immunocompetence (Hawley et al. 2009; Kilgas et al. 2010), recent studies suggest that the reaction to the PHA injection might be a nonspecific complex inflammation connected with massive infiltration of cells representing both adaptive and innate immunity

(Martin et al. 2006; Sarv and Hõrak 2009; Vinkler et al. 2010; 2012; Salaberria et al. 2013). Therefore, the PHA-induced swelling may be a multifaceted index of cutaneous immune activity, and we used this test because we were interested in a standardized index of immunocompetence (Salaberria et al. 2013), independent of the type of immune cells involved. We measured the immune response of lizards by using the PHA injection test (Smits et al. 1999). We used a pressure-sensitive spessimeter to measure thickness (to the nearest 0.01 mm) at the same point of the right hindlimb foot pad before and 24 h after injecting 0.04 mg of PHA dissolved in 0.02 ml of phosphate buffered saline (PBS) at the marked point. We calculated the immune response as the difference between pre- and post-injection thickness measures (Smits et al. 1999). The only appreciable effect of the PHA injection was a slight swelling of the skin, due to the immune response, which disappeared after 48 h. No lizard showed any sign of stress or pain due to this test.

Fig. 7 Above: PHA injection to the footpad of a lizard. Below: measurement of the footpad tickness

To activate the immune systems of male lizards, we used lipopolysaccharide (LPS) from the cell wall of the bacteria *Escherichia coli* (serotype 0111-B4; Sigma-Aldrich). LPS induces an inflammatory response by non-specifically activating B and T lymphocytes and producing specific anti-LPS antibodies but has no pathogenic effects (Janeway et al. 2001), although LPS also induces immunopathological effects such as fever (Deen and Hutchison 2001). Immune challenged male lizards were injected intraperitoneally with LPS (2.5 µg/g of body weight) diluted in 0.05 ml of phosphate buffered saline (PBS). The serotype was similar and the concentration similar or lower than those previously used to stimulate the immune system in other lizards (Deen and Hutchison 2001; Uller et al. 2006). Control males were injected with the same volume (0.05 ml) of PBS alone. Lizards were assigned at

random to the treatments and were handled “blind” by the same investigator using the same protocol for injections.

Parasite burden

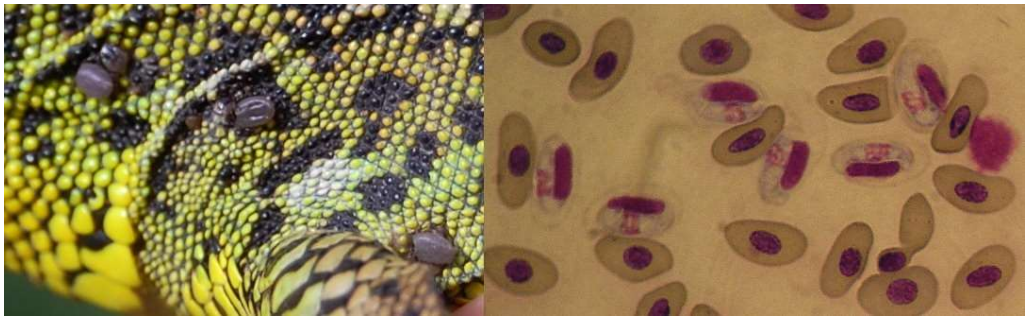

Fig. 8 Investigated parasites of *L. schreiberi*: ticks (*Ixodes* sp., left side) and haemogregarines inside the red blood cells of lizards (right side).

We counted the number of ectoparasites (*Ixodes* sp., Ixodidae) fixed on each individual lizard at capture time. Furthermore, we counted haemoparasites by using blood smears. A smear was made on a microscope slide from blood taken from a large subcutaneous vessel on the ventral side of the body by using a 25-G insulin injection needle and a 9-µl heparinized hematocrit tube. Blood smears were air-dried, fixed in absolute methanol for 10 min, and then stained in Giemsa diluted 1:9 with phosphate buffer (pH 7.2) before their examination for parasites. On mounted slides, half a smear, chosen at random, was scanned entirely at 200x along the length of the slide, looking for extraerythrocytic protozoa (Merino and Potti 1995). Numbers of intraerythrocytic parasites were estimated at 400x by counting the number of parasites per 2,000 erythrocytes. The only haemoparasites we found were haemogregarines. Haemoparasite prevalence was estimated as the percentage of infected lizards. Parasite median intensity was estimated for each infected host as the percentage of infected red blood cells found in approximately 2,000 cells.

Eggs and hatchling husbandry

Fig. 9 Eggs (left side), a hatching (middle) and a hatched offspring (right) of *L. schreiberi*.

Females laid their eggs in their own containers (between 27 June and 24 July) and eggs were immediately transferred as clutches to closed plastic boxes (13x13cm) filled with moistened perlite (1 g perlite: 1 g water). Plastic boxes with eggs were placed in an incubator at 27.5 °C (IRE-160; 94x60x60 cm; Raypa). Eggs were monitored every two hours between 0800-2200h, when hatching time was close estimated by condition of eggs. Immediately after hatching (between 20th August and 13th September), the hatchlings were measured with digital calipers (to the nearest 0.1 mm) (snout-to-vent length (SVL), head sizes) and body mass determined with a digital scale (to the nearest 0.01 g). Body condition was estimated using the residuals of the regression of log-body mass on log-SVL at hatching. We observed in some of the offspring morphological abnormalities of feet and tail. The absence/presence of undeveloped or curved tail and of supernumerary toes in each of the four feet were noted to made an index of "abnormality degree". After measurement, offspring was placed in outdoor 51×36×28 cm PVC terraria in the same conditions as females for further observations.

Fig. 10 Malformations of the offspring.

Results and Discussion

Chapter I. Field experiments revealed strong and complex environmental influences on lizard escape behavior in an emergency situation, but surprisingly, we did not find any effect of sex or reproductive condition/mating season. Moreover, preventive risk-taking behavior was not affected by any environmental variable. In spite that lizards have a likely higher detectability and that the attack success by a predator is higher in open rocky substrates than in dense vegetation, it seems that distance to refuge is not strategic in this lizard species. Depending on the microhabitat, we observed two different escape tactics. In grassy substrates where vegetation offered protective cover, flight initiation distance (FID) varied positively with the refuge distance, whereas FID was not related to distance to refuge in rocky substrates without cover. The habitat-dependent escape strategies might be explained by differences in availability of potential refuges or in detectability and attack success by a predator. Also, thermal differences between exposed and refuge locations in different microhabitats, and their associated thermal costs, might be important (Martín and López, 1999a, 2000a, 2010b).

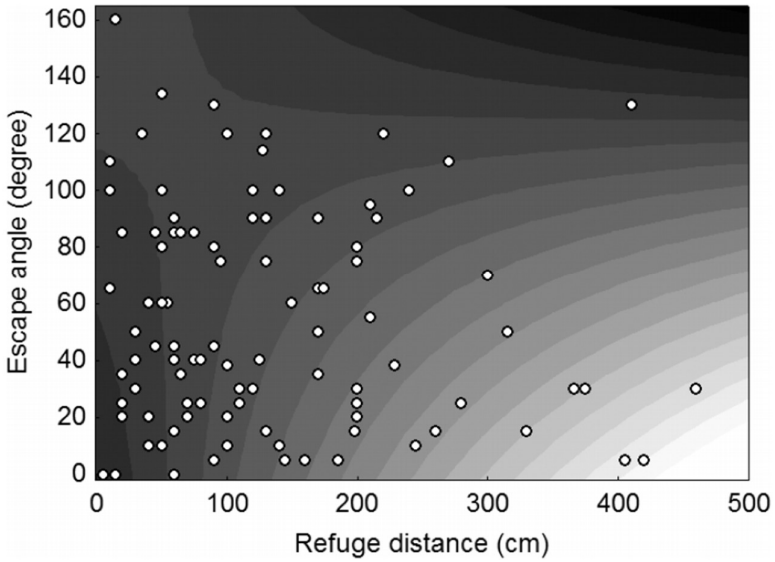

Figure 11. The effects of refuge distance, and escape angle on flight initiation distance (FID) of *L. schreiberi* lizards (shown in the *z*-axis with a colour code). The colour codes represent different flight initiation distances (FIDs) ranging from 0 cm (black) to 450 cm (white).

The relationship between escape angle and refuge distance affected FID in a complex manner. Lizards very close to refuge allowed the predator to approach closer than those farther from refuge, irrespective of escape angle. However, escape angle affected the FID of lizards farther from refuge. If the escape angle was large, the lizard allowed the predator to approach closer, irrespective of the refuge distance. For lizards farther from refuge, FID depended on refuge distance only if the escape angle was small. These results were expected because if a lizard is close to refuge, it can delay escape and still reach the refuge safely ahead of the predator even if the lizard has to flee toward the predator to get to refuge. If the escape angle is small (i.e., the lizard flees nearly directly away from the predator), lizards should adjust their FID depending on refuge distance (Cooper, 1997b; Stankovich and Blumstein, 2005). However, if the escape angle is large and, thus, fleeing may move the prey closer to the predator, one would intuitively expect prey either flee early when the predator is still far away or rely on crypsis and remain motionless as long as possible. Therefore, it seems that *L. schreiberi*, instead of taking the risk of running towards the predator or to an alternative refuge, let the predator approach closer while relying on crypsis as long as possible. By doing so, lizards accept a decreased margin of safety (Kramer and Bonenfant 1996) in exchange for a decreased probability of being detected and attacked. This tradeoff is highly plausible because most individual *L. schreiberi* lizards finally ran directly to the known refuge even if the predator was in that direction, which could still be safer than escaping to an unknown alternative refuge where they may face dangerous encounters with another type of predator (e.g., saurophagous snakes)(Amo et al. 2003, 2005).

The lack of intersexual differences in escape decisions is very interesting. However, other kind of compensations are still possible, e.g. running speed differences between gender (Cullum 1998; Lailvaux et al. 2003). Furthermore, the lack of gender effect could be explained by opposite constraints. Cryptic coloration of female *L. schreiberi* does not change seasonally, so females can always use the same effective crypsis and an associated short FID. In contrast, during the mating season coloration of males is more conspicuous, which should initially require longer FID, but conflicting reproductive requirements (i.e., mate

searching, territorial defense, mate guarding, etc.) may force males to be more active and take more risk by adopting shorter FID (Magnhagen 1991; Cooper 1997b, 1999; Cooper and Wilson 2007). Finally, after the mating season, there are no reproductive constraints and coloration of male *L. schreiberi* changes to being less conspicuous, which may allow males to be bolder and have shorter FID. Alternatively, only variation in environmental factors might be the determinants of escape behavior in this species.

Chapter II. The results of the experimental diet supplementation indicated that both carotenoids and vitamin E in the diet affected the expression of visual and chemical signals in *L. schreiberi* lizards. However, different traits were differentially affected, and in most cases, the addition of vitamin E had a higher effect on the expression of ornaments than the addition of carotenoids alone, even for those ornaments that are carotenoid-dependent.

In spite that structural throat coloration is not carotenoid-dependent, we found that both carotenoids alone and vitamin E alone increased brightness compared with control lizards, but the brightest throat coloration was observed in males supplemented with a combination of carotenoids and vitamin E together. However, the chromatic characteristics of throat coloration (i.e. hue and saturation) were not significantly affected by the supplementation. Change of throat brightness can be influenced both by melanin content and nanostructure (Megía-Palma 2016). Since UV and blue coloration were not altered, this might imply that melanin content did not change. Likely, nanostructural alteration could be the cause that increased brightness. Environmental stress may perturb the biochemical reactions that produce the light scattering structures responsible of structural colors (Kemp and Rutowski 2007; Mäthger and Hanlon 2007). Therefore, males with more antioxidants, such as carotenoids and vitamin E, may experience less physiological stress, allowing them to produce and/or maintain the regularity and spacing of structural components within iridophores, resulting in increased throat brightness. In fact, iridophores account for chromatic variation of carotenoid-dependent coloration in *L. vivipara*, which may be mediated by the non-pigmentary vitamin A_1 (San José et al. 2013).

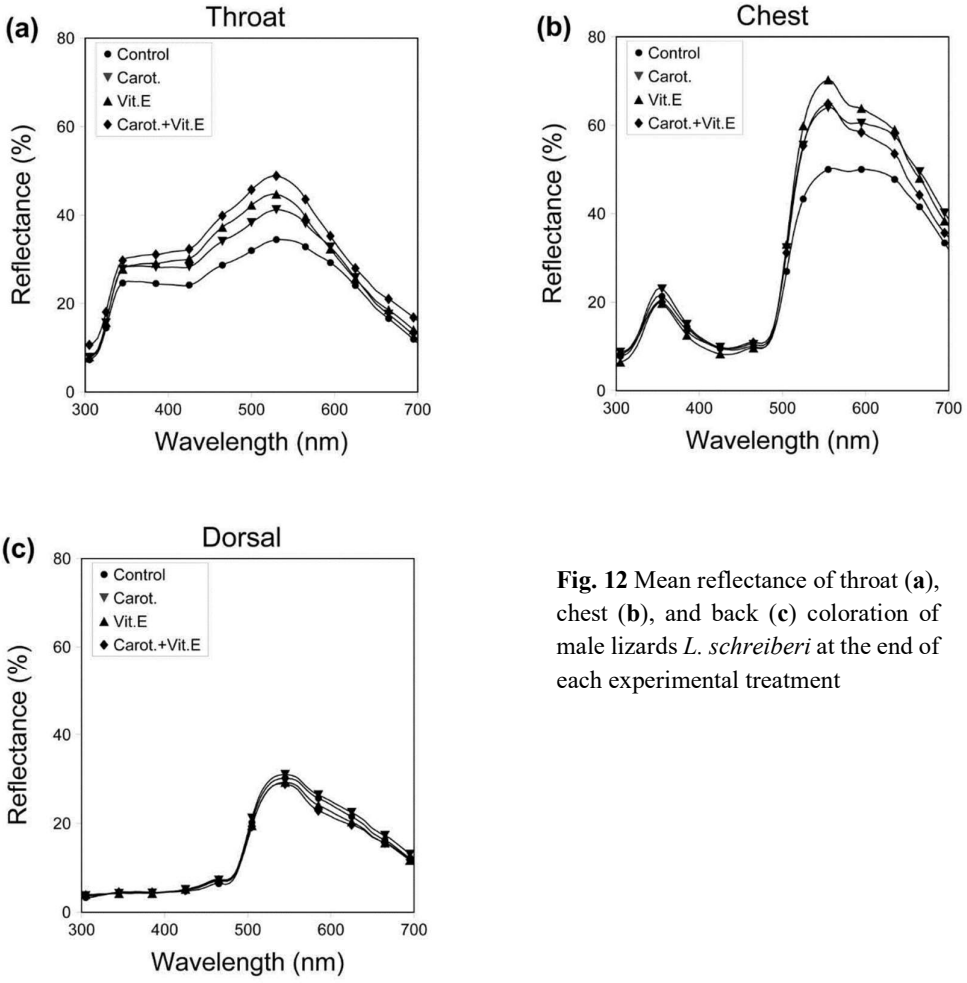

Fig. 12 Mean reflectance of throat (**a**), chest (**b**), and back (**c**) coloration of male lizards *L. schreiberi* at the end of each experimental treatment

Carotenoid supplemented lizards had more saturated "yellowish" chests than control lizards, but interestingly, lizards supplemented with vitamin E had even more saturated chest "yellowish" coloration than lizards supplemented with carotenoids alone. Surprisingly, the chest coloration of males provided with a combination of carotenoids and vitamin E was lower than when only vitamin E was supplemented, maybe caused by a possible competition for the same intestinal membrane transporter between carotenoids and vitamin E in the process of intestinal absorption (Reboul et al. 2007). Vitamin E supplementation alone and also with carotenoids increased UV and yellow coloration (300-390 nm and 580-700 nm wavelengths respectively), while carotenoid supplementation alone did not affect coloration. Probably, the carotenoid supplementation increased carotenoid incorporation into the xanthophores, while the effect of vitamin E was not only to support this incorporation and

defend carotenoids from oxidation, but also to cause nanostructural alterations in reflective layers resulting in UV increasing in chest coloration.

Interestingly, the supplementation of vitamin E or carotenoids, alone or in combination, did not affect the dorsal green coloration of male *L. schreiberi*.

The chemical composition of femoral secretions also varied between treatments; lizards supplemented with vitamin E alone or combined with carotenoids had different chemical profiles than lizards supplemented with carotenoids alone and control lizards. Supplementation of vitamin E in teh closely related *L. viridis* had a similar effect in femoral secretions (Kopena et al. 2011). These results support the hypothesis that an increase in dietary nonpigmentary antioxidants will be directly reflected in the femoral secretions. Because female *L. viridis* seem to be attracted to areas scent-marked by males with more vitamin E in secretions, it was suggested that this compound may act as an honest signal of male quality (Kopena et al. 2011). Vitamin E in secretions may signal the ability to obtain sufficient antioxidants, such that only males of high quality could allocate large amounts of vitamin E to the femoral secretions without causing a concomitant reduction in their antioxidant capacity (Brigelius-Flohe and Traber 1999; Martín and López 2010c; Kopena et al. 2011).

In **Chapter III** we found that vitamin E supplementation increased the PHA skin-swelling immune response, while the carotenoid supplementation did not influence it in comparison with the control treatment. Therefore, this experiment may support the hypothesis that carotenoids are not effective immunostimulants per se, at least in this lizard species and with respect to the aspects of the immune response measured by the PHA test. Many other studies of birds and fish also show a lack of effects of carotenoid supplementation on the immune response (Navara and Hill 2003; McGraw and Ardia 2005; Biard et al. 2006; Hõrak et al. 2006, 2007; McGraw and Klasing 2006; McGraw et al. 2006; Lin et al. 2010; Sutherland et al. 2012). However, since the PHA test does not show the whole immune response of the body, we have to narrow our conclusions. Vitamin E

supplementation had a clear increasing effect in PHA-induced immune response, having similar immunostimulatory effects as it has been observed in domesticated animals (Surai 2002). This suggests that the cost of allocating antioxidant and inmunostimulatory vitamin E to secretions may confer reliability to chemical signals of green lizards.

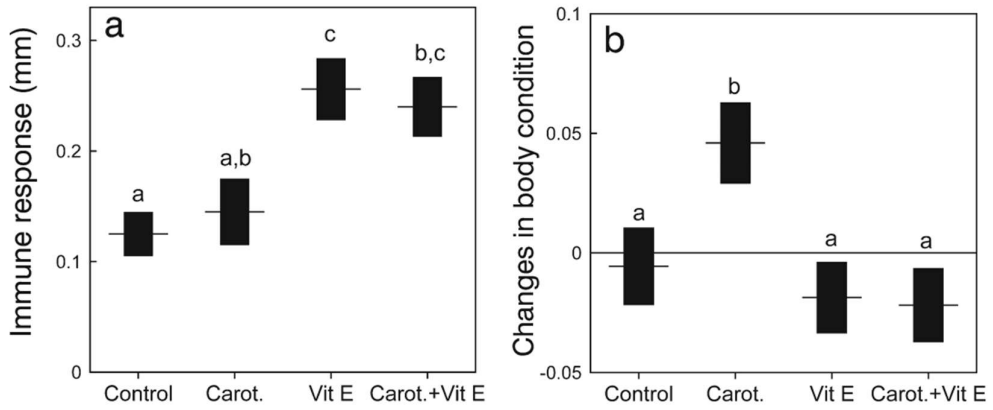

Fig. 13 PHA-induced immune response (**a**)(X ±1 SE; in millimeter) and change in body condition (**b**)(X ±1 SE) of male lizards *L. schreiberi* in each experimental treatment. Means with the same letter above the bars were not significant different.

Lizards supplemented with carotenoids plus vitamin E combined had greater immune responses than control lizards, but these responses only tended to differ marginally of lizards supplemented with carotenoids alone. This is an interesting result, because these two antioxidants may have synergistic effects (Leibovitz et al. 1990; Palozza and Krinsky 1992; Surai et al. 2001a). However, this result might be explained if carotenoids required vitamin E against oxidation or by the competition elicited by the similar intestinal absorption mechanism of carotenoids and vitamin E (Woodall et al. 1996; Surai 2002, Reboul et al. 2007).

Surprisingly, body condition of lizards increased only after the carotenoid supplementation, while vitamin E supplementation alone or with carotenoids decreased body condition. This suggests that carotenoids may have some important role affecting positively to health state and condition of lizards, possibly as antioxidants. However, changes in body condition were not related to differences in the immune response. In some growing nestlings bird species, carotenoid supplementation may also increase body condition by regulating

oxidative stress resulting from rapid growth (Biard et al. 2006). Interestingly, the positive effect of carotenoids disappeared when carotenoids were given together with vitamin E, suggesting that dietary vitamin E and carotenoids may interact with each other in a complicated fashion.

In **Chapter IV** we found that vitamin E increased saturation of UV-blue throat coloration but not brightness. A possible reason for changes in throat coloration can be nanostructural changes in platelet distances and spacing. However, an increase in melanin-content may be more likely, because the changes in coloration observed here agree with the changes found in a study where melanin-content of *L. schreiberi* skin was decreased artificially by oxidation (Megía-Palma et al. 2016). In this previous study, it was found that melanin extraction from throat blue skin increased brightness and decreased short wavelength of the spectrum relative to long wavelength. In our study, it was observed the opposite process, brightness decreased and short wavelengths increased, possibly due to melanin increased. Melanin production occurs in several different ways. One possible way is by reduction of glutathione level, which is the most important intracellular antioxidant (Anderson 1998; Wu et al. 2004). Thereby, when glutathione level is high, the melanin production is inhibited, but when glutathione level is low, melanin production is started in melanocytes. But, with decreasing glutathione level, the body requires another effective antioxidant (vitamin E in our study) to substitute the antioxidant effect of glutathione in the cells, otherwise, the lack of alternative antioxidants would infer very high oxidative stress (Halprin and Ohkawara 1966; Benedetto et al. 1981; Galván and Alonso-Alvarez 2008). The immune challenge alone also increased throat UV-blue saturation, which was also likely due to increases in melanin. This could be explained because histamine, which is released as the first reaction of the body in case of an immune activation, and nitrogen-oxide, which is released by macrophages and neutrophils after the immune activation by LPS (Tsatmali et al. 2000), have positive effects on melanin production, at least in mammals (Sasaki et al. 2000; Yoshida et al. 2000). Free radicals also can decrease glutathione resulting in melanin increasing in melanophores. However, without substituting antioxidants, the body would suffer heavy oxidative stress with heavy costs for lifetime. Thus, cheating in melanin based

signals can be very costly. When there is a lack of other alternative antioxidants, it is worth to maintain high glutathione levels, resulting in less intensive melanin-based coloration (Galván and Alonso-Alvarez 2008). Other possible explanation for this surprising response to the immune challenge is the genetical link between melanin production and immunity, e.g., pleiotropical genes can affect simultaneously melanin-based coloration and immunity (Roulin 2015). However, the immune challenge also increased very short UV wavelengths of throat coloration, which may be explained by nanostructural changing in iridophore layers or maybe by neural and hormonal changes (Teyssier et al. 2015). The effects of the immune challenge and vitamin E supplement were additive, the group with both manipulations (E+L) had the "highest" UV-blue coloration.

The immune challenge alone did not affect chest coloration, but when it was combined with a supplement of vitamin E it had a strong effect, decreasing yellowish saturation. Immune challenge likely increased free radical level and carotenoids were bleached. Thus, the supplement of vitamin E was not useful or was not used for defending carotenoids from oxidation.

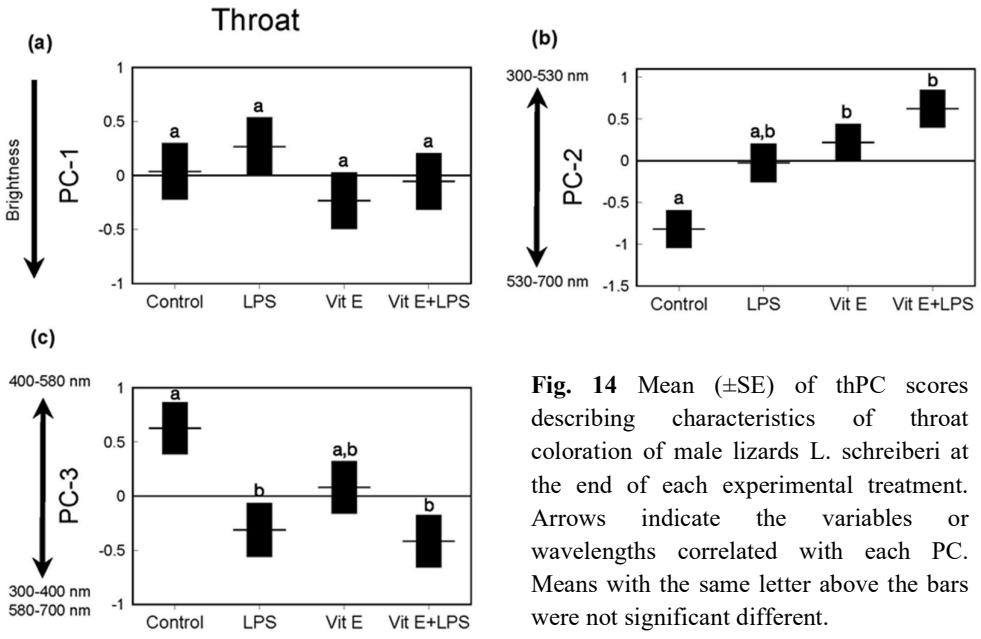

Fig. 14 Mean (±SE) of thPC scores describing characteristics of throat coloration of male lizards L. schreiberi at the end of each experimental treatment. Arrows indicate the variables or wavelengths correlated with each PC. Means with the same letter above the bars were not significant different.

Dorsal coloration changes were similar to those of chest coloration but more defined. We found that vitamin E alone increased brightness but decreased green saturation and carotenoid chroma. Immune challenge alone did not have effects on dorsal coloration, but when it was combined with vitamin E, brightness increased and green saturation and carotenoid chroma decreased, likely because carotenoid content of xanthophores decreased.

Body condition showed a closely significant pattern, vitamin E supplemented groups had lower values. Vitamin E needs lipids to be absorbed and transported in the body, and, furthermore, the color alterations observed in groups with vitamin E also need energy. Therefore, males might utilize the energy acquired from food during the experiment to make these changes. It seems that the immune challenge did not affect fat stores- Nonetheless several earlier studies found that immune activation by LPS elicited anorexia, higher glucose metabolism, and, furthermore, decreased activity (Feingold and Grunfeld 1992; Huang et al. 1999; Dantzer 2001). However, in a study in laboratory environment where animals were fed, this fat store decreasing effect disappeared due to the availability of more food than would be consumed in a natural habitat under a sickness condition (French et al. 2007; Ruiz et al. 2011).

With respect to chemical signals, vitamin E content in femoral secretions increased in the two vitamin E treatment groups. Interestingly, the increase of vitamin E in secretions occurred independently of that lizards had or had not been immune challenged.

Together interpreting changes of signals, we can see that dietary vitamin E provides an increase in melanin-based coloration in throat against a decrease in carotenoid-pigment coloration of chest and back. However, the increasing in brightness and the UV range can have positive effects on conspicuousness of signals for females. Therefore, vitamin E supplemented lizards allocated energy for throat coloration, which may be the most important visual signal for *L. schreiberi* as it occurs in closely related lizards (Bajer et al. 2010; Bajer et al. 2012; Molnár et al. 2012) and which may be confirmed by the iridescence nature of throat coloration (Pérez i de Lanuza and Font 2014) requiring more precise nanostructure organization and increasing production and maintenance costs (Doucet and

Meadows 2009). On the other hand, even if the death of one challenged male suggested that the immune activation was a relevant challenge for the body, challenged males surprisingly also increased the intensity of sexual signals. Males with an immune challenge might be trying to maximize future fitness by increasing current signal intensity in a situation where long term expectatives of survival and future reproduction are low. Similar results were found in common lizards (*Lacerta vivipara*); male quality signaled by red ventral coloration increased by the effect of corticosterone, which activates physiological stress response and showed positive correlation with lipid peroxidation level in males (Cote et al. 2010). However, why the immune challenge alone did not affect carotenoid-based signals is still unsolved. In goldfinches (*Carduelis tristis*) (Navara and Hill 2003) and in great tits (*Parus major*) (Fitze et al. 2007) it was also found a lack of effects of immune challenge on feather carotenoid accumulation, however, the reason might be that feathers are not able to change rapidly their carotenoid content (Toomey et al. 2010).

The most relevant alterations of coloration were observed in the group immune challenged and supplemented with vitamin E. We found similar effects than in the vitamin E alone group, but more intense. This can be explained by the same effects than in the immune challenged alone group, but in the combined treatment males would have an additional source of vitamin E to increase femoral secretion and to act as an effective antioxidant to substitute glutathione in visual signals. In contrast, the price of a higher throat color intensity seems to be the reduction of carotenoid level in other body parts that, nevertheless, seems to be less important as sexual signals in these lizards. However, the increasing of brightness and UV of other visual signals can help females to perceive males by contrast with the environment more easily and this can help to increase mating possibilities.

Seeing together results of **Chapter II-IV**, there are some surprising differences in the effect of vitamin E for development of sexual signals. Although vitamin E supplementation increased vitamin E content of femoral secretion in all years, the visual

sexual signals reacted in different ways in different years. In the experiment of **Chapter II**, made in 2011, we found that vitamin E increased throat brightness, but not UV-blue saturation, whereas, in the study of **Chapter IV**, made in 2013, vitamin E supplementation increased saturation of UV-blue coloration but not brightness. However, it could be possible that the increase of melanin elicited a reduction in brightness that hid the positive effect of vitamin E for brightness. There were also some differences in the effects on chest coloration; in 2011, vitamin E supplementation increased yellow saturation, carotenoid chroma and UV reflectance of chest coloration, but not the brightness, while in 2013, brightness was increased by vitamin E treatment, but not yellow saturation and UV reflectance, while carotenoid chroma decreased. In this latter year, supplement of vitamin E might not be useful or might not be used for defending carotenoids from oxidation. These differences show that vitamin E decreased carotenoid-content of the skin in the latter study but increased it in the former study. Dorsal coloration was not affected by vitamin E treatment in **Chapter II**, but it was much more affected in **Chapter IV**. In 2013, we found that vitamin E alone increased brightness but decreased green saturation and carotenoid chroma.

A possible reason of these important differences between experiments is the difference of weather conditions between the two years. The study of **Chapter IV** was made in a very cold and cloudy spring ($T_{average\text{-}May2013}$: 9.3 ºC), while the study of **Chapter II and III** was made in an "average" warm spring ($T_{average\text{-}May2011}$: 13.3ºC while $T_{average\text{-}May2009\text{-}2015}$:12.5ºC). The different temperature and sunbathing (i.e., basking) possibilities may change the structural development and melanin production of the skin. Furthermore, due to the different environmental circumstances, it is possible that females might prefer different male signals according to the availability of food or raw elements of signals. In a cold year with few sunny days, structural throat coloration can be a more important signal because UV intensity is highly affected by temperature (Bajer et al. 2012) and likely sunbathing. Furthermore, suboptimal body temperature can increase free radical levels of the body in males (Ballen et al. 2012). This signal can indicate a good quality home range with good sunbathing possibilities and less developmental stress (Kemp and Rutowski 2007; Mäthger and Hanlon 2007). Flexible female choice is not a rare phenomenon, however less

investigated (Kahn et al. 2013). In lark buntings (*Calamospiza melanocorys*), females shift preferences on male traits across years to increase their reproductive success (Chaine and Lyon 2008). In collared flycatchers (*Ficedula hypoleuca*), development of different sexual badges was influenced by fluctuating climate conditions in different climatic areas (Garant et al. 2004; Hegyi et al. 2007). Flexible mate choice in *L. schreiberi* might allow females to choose a male character that will maximize her fitness in the environmental conditions of each year. This adaptive plasticity in female mate choice can explain the evolution and maintenance of multiple signals in this species.

Chapter V. We found that males with traits related to a higher dominance status (i.e., relative bigger head size or more intensive UV-blue throat coloration) use larger home-ranges that overlap with more males and overlap more home ranges of other dominant males with more intensive UV-blue throat coloration, whereas males with smaller home-ranges avoid these dominant males. Movement on larger home range area entails overlapping home ranges of more males, likely increasing the frequency of agonistic encounters with these males. Males with bigger heads have stronger bite (Perry et al. 2004; Huyghe et al. 2009) increasing their fighting ability. Hereby, males with bigger heads gain an advantage in agonistic encounters raising their status in social dominance. Factors influencing relative head size are unclear, but the early life development of the head can be influenced by testosterone, however there is no direct evidence (Vitt and Cooper 1985; Sassoon et al. 1987; Perry et al. 2004; Huyghe et al. 2009). Intensive UV-blue coloration of the throat is a basic dominant status signal in Iberian and also in European green lizards (Martín and López 2009; Bajer et al. 2011). Throat coloration is structural and melanin-based, reduction of melanin content in the skin decreases UV and blue color of skin (Megía-Palma 2016). Testosterone can increase melanin-based social badges (Evans et al. 2000; Gonzalez et al. 2001), furthermore can provoke a more aggressive behavior (Wingfield et al. 1987), which frequently associates with a higher activity and a larger home range size (DeNardo and Sinervo 1994). Although it is not proven that throat patch coloration is a testosterone directed

social signal, this would explain a higher movement activity and a higher dominant status by aggression, because intensity of this patch did not correlate with relative head size, nor condition or body length which could provide any advantage in an aggressive encounter.

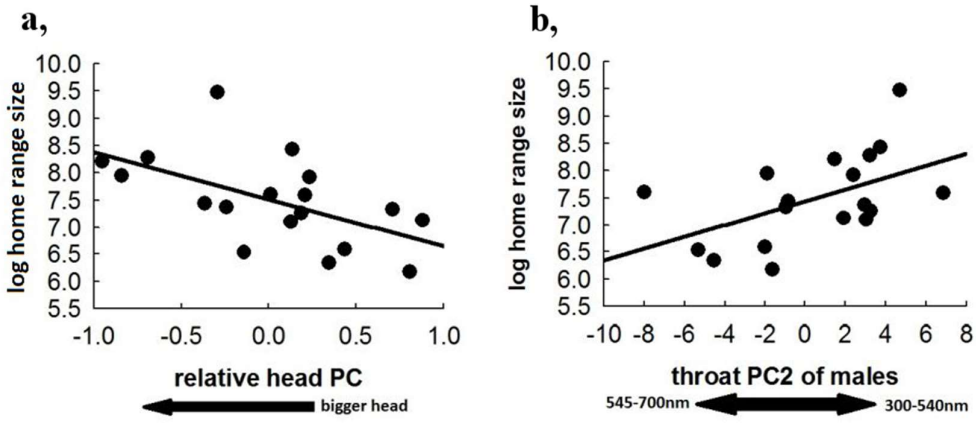

Fig. 15 Relationships of relative head size (**a**) and throat PC2 (**b**) with log home range size in male *L. schreiberi*.

Males overlapping with females seemed to show two different reproductive strategies according to the different strategies of space using. Large home range user males overlap more females, likely trying to increase reproductive success by mating with a higher quantity of females by increasing the number of encountered females (Marco and Pérez-Mellado 1999; Haenel et al. 2003) or by increasing forced-copulations due to dominant traits. However, females do not always prefer dominant males (Qvarnström and Forsgren 1998), cryptic mate choice can allow to females to select mates (Olsson and Madsen 1998). Males with less dominant traits, have smaller home ranges and rather invest in mate guarding of a good quality female. The smaller their home ranges are, the more UV-green back overlapping females are, which can mean a strict mate guarding. In other study, we found that females with more UV-green back are bigger and have less parasites, and, furthermore have faster developing embryos in eggs (Chapter VI). Mating with a high quantity of females or with less females but of high quality can lead to similar reproductive success.

Investigating space using of females, we found that there is a negative relationship between home range size and saturation of female back coloration. A smaller home range size may be concomitant with lower predation risk and lower stress, which can increase saturation of back coloration in females of this species (Chapter VI). Females with more saturated backs have more saturated yellow chests too, which can support they are affected by lower stress level because carotenoids react with stress provoking free radicals (Krinsky and Yeum 2003).

Females with larger home ranges overlap with more males, suggesting increased mating possibilities, but females with smaller home ranges overlap with less males but these males have a high content of α-tocopherol in femoral secretion. Interest of females in a polygynic system to mate with as more and better quality males as they can do. Overlapping with more males can imply more matings and a high possibility for cryptic mate choice to select the best sperms from the best males. But if females are chosen by a mate guarder male and avoid of guarding can be too energy wasting, females can accept this male as pair. But losing the advantage of cryptic mate choice, females may need to estimate quality of males and also the importance of sperm condition is higher in a mate guarded situation due to a lower number of mating partners. Appropriate estimate of sperm condition increases the possibility of successful reproduction. Sperm condition is strongly influenced by antioxidants, α-tocopherol has important roles to avoid infertility by maintaining sufficient quantity, viability and motility of spermatozoa with unfragmented DNA and free-radical resistant, polyunsaturated fatty acid rich plasma membrane (e.g., Suleiman et al. 1996; Surai et al. 1997; Keskes-Ammar et al. 2003; Greco et al. 2005; Eid et al. 2006). Females can prefer males with higher α-tocopherol content in femoral secretion to ensure their own reproductive success and increase attractiveness of their offspring. A previous study supports our results, we found a female preference for areas scent marked with high levels of α-tocopherol in *L. viridis* (Kopena et al. 2011). This confirms that α-tocopherol has a relevant role in mate choice of *L. schreiberi*.

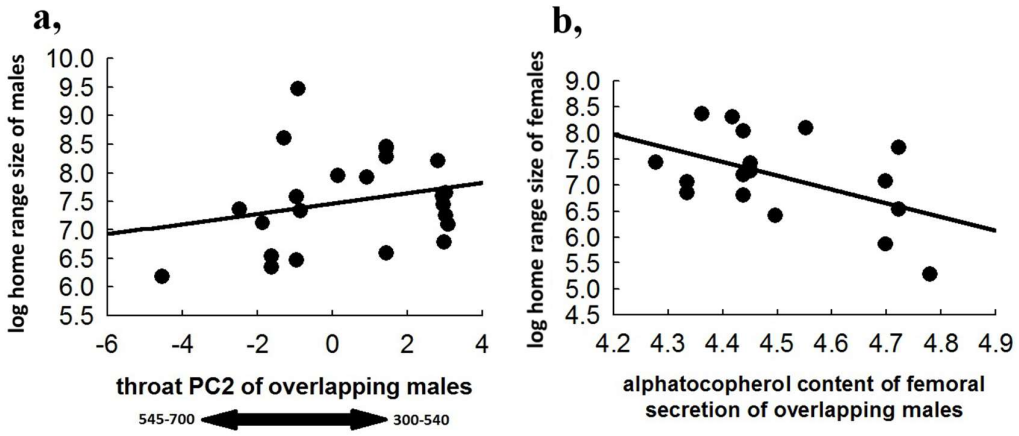

Fig.16 Relationship of male home range size and median of throat PC2 values of their overlapping males (**a**). Relationship of female home range size and median of α-tocopherol content in femoral secretion of their overlapping males (**b**).

Researching female-female overlapping, we found that females with smaller home range areas overlap with less females, but space using of females with larger home ranges are influenced by the traits of overlapping females. If overlapping females have more UV-green backs, females reduce their home range area comparing to when overlapping female are browner. A possible reason can be that females with more UV-green backs attract high quality males into their home ranges and it could be rather worth to reduce home range areas and try to copulate with the high-quality males of the more UV-green females than investing energy moving on larger home range areas to copulate with a high number of males.

The different reproductive strategies of males and behavioral adaptation of females to these strategies can explain the similar home range sizes of gender and support the mixed evolutionary stable strategies (mixed ESS; Maynard Smith 1982; Számadó 2000). However, traits of attractiveness may change between years due to different environment and possible flexible female mate preferences (unpublished result, Chapter IV), but the basic dominant-attractive strategies of males can be permanent resulting in similar reproductive success for all the males.

Chapter VI. Comparing coloration of males and females, we found that males have more saturated UV and blue coloration on throat and greener backs. Females are brighter on

all body parts and surprisingly, they have more yellowish chests with higher carotenoid chroma than males.

Cost of vitellogenesis have serious effects on condition, but not on the immune response. Mother condition after egg laying decreased by increasing relative clutch size. This actual investment can increase actual reproductive success but can decrease the future reproductive success, such as in *Lacerta vivipara* (Bleu et al. 2011). After egg laying, the PHA-based immune response was positively depended only on mother size which is a frequent phenomenon (see e.g., Tobler et al. 2011; Vinkler et al. 2012) that maybe caused by larger animals having larger cellular reserves and larger volume of skin prone to inflammation (Vinkler et al. 2012).

Coloration of mothers show health condition after egg laying. Females with more intense UV and blue throat coloration have less parasites in blood, which can be caused by the positive effect of antioxidants on arrangement of reflective platelets in iridophores. Haemoparasites can decrease antioxidant level of the body in a complex way: (i) blood parasites can elicit physiological stress reaction (Tomás et al. 2005), (ii) increase lipid peroxidation in the blood (Commins et al. 1998, Deger et al. 2009, Saleh 2009), (iii) acut phase of immune reaction can decrease carrier molecules of lipid soluble antioxidants (Das et al. 1996). Mothers with more intense UV and green dorsal coloration are bigger and have less ticks. However, likely, green back coloration is a genetically determined trait, maybe green backed females have a better carotenoid absorbing intestinal system and allocation into the skin, but with more carotenoids they have a more effective parasite resistence. Furthermore, bigger sized females had more yellow chest coloration. This phenomenon is not rare (see Freeman-Gallant et al. 2014; Jawor et al. 2004), bigger (and likely older) females may increase carotenoid intake by a longer life experience. A better carotenoid supply can contribute to develop a stronger immune response in bigger females, both in case of females with more yellowish chests or greener backs, because carotenoids have important roles in the immune system (Chew and Park 2004). However, carotenoid supplementation did not increase the PHA-induced immune response of males in *L. schreiberi* (Chapter III).

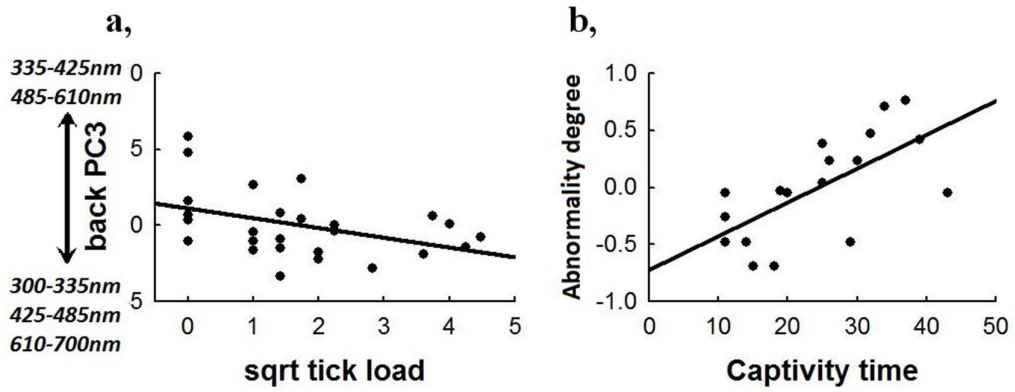

Fig. 17 Relationship of tick load and back PC3 of females (**a**). Relationship of captivity time of females and average abnormality degree of their eggs (**b**).

Number of laid eggs was determined only by mother size, parasite burden did not affect absolute or body size-relative clutch size. Similarly, we did not found effects on the number of hatched offspring, nor on hatching success. Date of egg laying and incubation time of eggs was not influenced by parasite burden, nor by condition.

Mother coloration did not predict relative clutch size, but females with more saturated back coloration had more hatchlings and higher hatching success. Furthermore, females with more saturated back coloration laid their eggs earlier. Chromatophoral background of higher saturation (higher dPC2: less 300-475 nm but more 475-700 nm) in green and brown coloration is less known. Special cooperation by production of more melanin absorbing more light and a higher background reflectance increasing long wavelengths can elicit higher saturation of green and brown dorsal coloration. Protein or calcium consumption can affect melanin production (reviewed in McGraw 2008), furthermore, they can improve development of eggs in the oviducts of females. Higher melanin content of the integument can also increase body temperature faster (Cloudsley-Thompson 1999; Reguera et al. 2014), increasing metabolism and maybe the rate of egg development too. Thus, females require less sunbathing time to reach appropriate body temperature, decreasing predation risk. However, a higher background reflectance can decrease this warming effect. Furthermore, alteration of precision in arrangement and thickness of reflecting platelets can have similar impacts on saturation. The higher the

reflectance is at middle (green) or long (brown) wavelengths and the lower the reflectance is at shorter wavelengths, the higher is the saturation of green or brown coloration. Arrangement of platelets in iridophores can be regulated by endocrine and nervous system, both can induce contractile activity of the actin filaments altering space of purine crystals, however, results are inconsistent in different species (Ligon and McCartney 2016). Furthermore, vitamin A_1 is related with ontogenetic changes in fish chromatophores (Miwa and Yamano 1999), thus, health state and carotenoid supply transforming vitamin A_1 may influence dorsal saturation and investment into reproductive success in *L. schreiberi*.

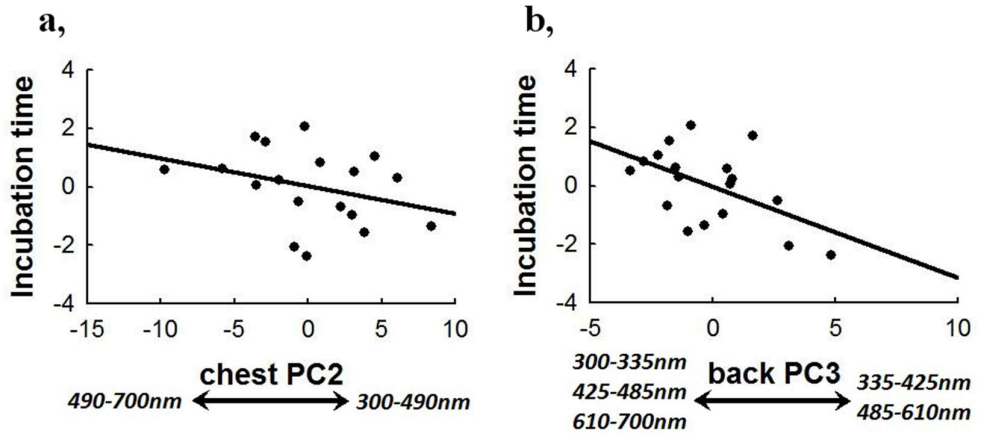

Fig. 18 Relationships of chest PC2 (**a**) and back PC3 (**b**) of females with average incubation time of their eggs.

Eggs from females with more yellowish chests have longer incubation times, however, greener dorsal colored females have a bit shorter incubation timed eggs. Longer incubation provides time to normal development for the embryo on stable temperature. Due to fast metabolism, free radical production rate is very high inside of the eggs, thus, embryos require large amount antioxidants for normal development (Blount et al. 2000; Surai et al. 2001a). Antioxidant capacity of carotenoids is higher under lower oxygen pressure, as it is found inside of the eggs (Surai et al. 2001b). Thus, carotenoids and other antioxidant investment into yolk can increase survival of the eggs and offspring quality. Furthermore, besides a large availability of nutrients can provide more time for appropriate development of the embryos inside the eggs, in a safe place. Carotenoid-rich *L. schreiberi* females can

invest more antioxidants into eggs resulting in longer incubation times. However, greener backed females have eggs with shorter incubation times, likely these embryos have faster developing rate. Based on our long-term field studies, adult females with green dorsal coloration likely have a bit faster growing rate than females with brown dorsal coloration (unpublished data). Maybe this genetic or carotenoid-based higher developing rate can be very beneficial for the embryo by hatching earlier and having more time for feeding and reaching good condition before hibernation. This is important because body size and condition can be the strongest selective factors for hatchlings for winter survival (Civantos et al. 1999; Civantos and Forsman 2000) in a cold mountain clime, partly, because hyperglycemic cryoprotective system can be the base of winter survival in *L. schreiberi*, such as in *L. vivipara* (Costanzo et al. 1995; Grenot et al. 2000).

Head height and width of offspring are strongly influenced by the mother head sizes and mother haemoparasite burden. Higher and wider headed offspring are able to consume bigger sized food eliciting a faster enhancing of body condition. Thus, the presumable heritable head sizes of mother can positively affect offspring development, whereas mother haemoparasite burden may decrease body increasing rate, thereby, influence negatively survival possibility, competitive ability and maybe mature time of offspring. Thus, it is worth for males to recognize and prefer parasite resistant females, because this can increase their future fitness.

Mothers with higher UV throat intensity and more saturated but - maybe because of more melanin - less bright dorsal coloration have bigger hatchlings. More saturated dorsal colored mothers also have longer headed offspring and more intensive UV throat colored females have wider headed hatchlings, however these relationships can be originated from body size correlations. Mothers that express a higher UV throat intensity can possess more antioxidants in their body because developmental stress (increasing free radical levels) have negative effects on structural coloration (Kemp and Rutowski 2007; Mäthger and Hanlon 2007). Furthermore, carotenoids and vitamin E can increase structural-based throat brightness of male *L. schreiberi* (Chapter II), thus, these females can invest more

antioxidants into egg yolk that promote offspring size (George et al. 2001; Biard et al. 2005). Moreover, females with more saturated, darker backs have bigger hatchlings too (see above reasons). More intense but less bright UV-blue throats and more intense but less bright chests of mothers can predict hatchlings with a better condition. Structural coloration also can indicate a better investment in reproductive success, such as in blue tits, where females with more intensive UV crown laid bigger eggs (Szigeti et al. 2007) and had more fledglings (Henderson et al. 2013). Furthermore, lower UV-blue throated *L. schreiberi* females have a higher amount of blood parasites and diseases can decrease carotenoid investment into egg yolk (Surai et al. 2001b). Furthermore, these females can possess more antioxidants to be able to afford more intensive structural throat coloration and a chest with a higher content of carotenoids.

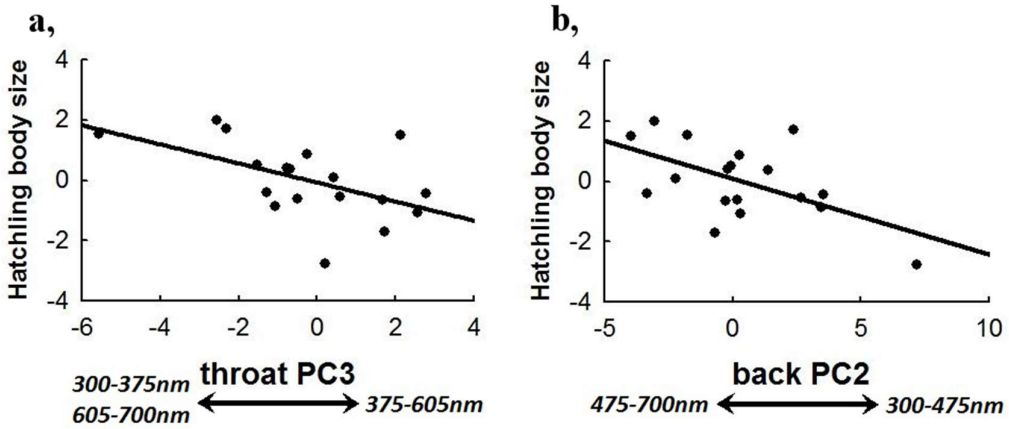

Fig. 19 Relationships of throat PC3 (**a**) and back PC2 (**b**) of females with average body size of their hatchlings.

Captivity, as a source of chronic stress, can influence reproductive success. Stress induced by the presence of predators can increase plasma corticosterone in birds (Silverin 1998; Scheuerlein et al. 2001). Increased maternal corticosterone enhance corticosterone content of egg albumen (Hayward and Wingfield 2004; Rubolini et al. 2005), which can provoke higher embryonic mortality, developmental instability of skeletal traits, impaired development, lower hatching body mass and slower growing of hatchlings (Mashaly 1991; Heiblum et al. 2001; Eriksen et al. 2003). We found, that longer captivity times of pregnant females resulted in a lower number of hatchlings and a lower hatching success, with

hatchlings of smaller average body size, head length and width at hatching, and, furthermore, these offsprings had more morphological abnormalities. The number of days in captivity negatively influenced back color saturation, likely by decreasing melanin content and iridophore reflectance of the skin. Malnutrition is a possible reason of melanin content reduction of the skin (reviewed in McGraw 2008). Maybe mealworms and crickets have less phenylalanine, tyrosine or calcium than the natural nourishment of *L. schreiberi* which might lead to lower melanin production. But corticosterone can also decrease melanin-based coloration by specific glucocorticoid receptors in melanophores, leading to melanosome aggregation within melanophores and/or reduction of melanin concentration (Greenberg 2002; Ducrest et al. 2008; Roulin et al. 2008). Like melanophores, iridophores have similar cell receptors that belong to the same receptor superfamily as glucocorticoids receptors (Evans 1988; Miwa and Yamano, 1999), which may influence spacing of iridophores or spacing of reflective platelets inside. Females of *Sceloporus virgatus* react to corticosterone with smaller and less colorful throat patches (Weiss et al. 2013). Surprisingly, this stress effect did not influence mother condition, nor immune response after egg laying, but corticosterone is an energy mobilizing hormone (Romero 2002; Moore and Jessop 2003) and long-term increased corticosterone levels by chronic stress have suppressive effects on the immune system (Dhabhar and Mcewen 1997; Dhabhar 2000).

In summary, we found structural, carotenoid and melanin-based coloration together indicate quality of both females and their offspring, supporting mutual mate choice, mainly in mate-guarder males. Since female signals seems to be honest, these indicator signals likely have a role in maintenance of *L. schreiberi* female coloration. But due to the similar base of coloration on different body part, intersexual genetic correlation is neither excluded. Moreover, these two phenomena would increase even synergistically the fitness of individuals that prefer these color signals.

Conclusions

- Antipredator experiments show that sex or reproductive condition/mating season do not affect preventive risk-taking behavior, nor active escape decision in an emergency situation. Thus, males do not apply behavioral compensation for higher risk in mate searching and conspicuous coloration. Antipredator strategies of *L. schreiberi* only depend on the physical environment and the relative situation of the predator and the refuge.

- Carotenoid supplementation is able to increase not only carotenoid-based, but structural visual signals. Thus, carotenoid supplementation increases carotenoid content of xanthophores, contradicting earlier studies, and, furthermore, it was able to change the spacing of reflective platelets in iridophores.

- Carotenoid supplementation does not affect the immune function in males of this species, at least not the PHA-induced immune response. The LPS-induced immune challenge has neither effects on carotenoid- (likely lutein-) based coloration. However, PHA and LPS-induced immune responses do not cover all the immune system and we have to be careful with conclusions. Nevertheless, carotenoids have relation with health by their effects on body condition (as fat stores). Carotenoid supplementation increases body condition, while a negative effect on body condition decreases carotenoid chroma on chest and back, suggesting that carotenoid signals indicate different types of quality than other visual signals.

- Immune challenged males increase intensity of some visual signal components, especially when combined with supplementary vitamin E that can boost sexual signals, increasing vitamin E content of femoral secretion and acting as an effective antioxidant to substitute glutathione in melanin-based signals. Males with an immune activation suffer a relevant challenge for the body and might be trying to maximize future fitness by increasing current signal intensity in a situation where long term expectatives of survival and future reproduction are low. In contrast, the price of the higher throat color intensity seems to be,

at least in the combined treatment, the carotenoid decrease in other visual signals that, nevertheless, seems to be less important as sexual signals in these lizards.

• While vitamin E supplementation shows a consistent vitamin E content increasing in femoral secretion in all treatments, we found conspicuous differences when comparing the effects of vitamin E supplementation on visual signals in two different experiments made in different years on the same population. Differences might be caused by different weather conditions that cause different challenges to males when developing sexual signals, which might be the base of flexible female choice. Therefore, multimodal signals of *L. schreiberi* can be part of a special back-up signal system. Vitamin E content of femoral secretion may be a stable signal about the antioxidant status, while simultaneously the health status might be related to one or more environmentally influenced visual signals. Which signal flexible indicate male quality would depend on which visual trait is more reliable showing quality under each given environmental conditions. This alteration in male sexual signals would suppose the existence of flexible female mate choice that can be the base of evolution and maintenance of multiple signals of *L. schreiberi*.

• Different strategies of space use may reflect different mating strategies of male *L. schreiberi* to which females are adapted. Different mating strategies of males can be evolutionary stable strategies which provide benefits both to mate guarder and dominant males. Similar reproductive success can maintenance honesty of signals and, furthermore, the multiple sexual signal system in this species.

• Females prefer males with higher vitamin E content in femoral secretion likely to increase fertility. Our results confirm that vitamin E has important roles in health state, at least in the PHA induced immune response and antioxidant system, which may indeed indicate good mate quality for females.

• Structural, carotenoid and melanin-based coloration of females together indicate different components of the females' health state and of their offspring number and quality. These indicator signals support the existence of mutual mate choice, especially in case of males with a mate guarding strategy, which, likely, may have a role in the evolution and

maintenance of *L. schreiberi* female coloration. However, due to the similar pigment and structural bases of coloration in different body parts in both gender, intersexual genetic correlation cannot be entirely excluded. Moreover, these two phenomena would increase, even synergistically, the fitness of individuals that prefer these color signals.

- Both the different space use and mating strategies of males, the environment-dependent flexible mate-choice and the intersexual genetic correlation may have roles in the evolution and maintenance of multiple sexual signals in *L. schreiberi*.

Future perspectives

This thesis gave responses to some questions but also showed some surprising and interesting results requiring some more explanations or opening some new possibilities of research of *L. schreiberi'*s multiple sexual signals.

- Although our results show that the different genders have no difference in antipredator strategy, it may be worth to research whether the different color morphs of females and different individual males with different bright visual sexual coloration differ in the antipredator strategies, because it might be that the lack of difference between males and females are not related to conspicuousness, only to the different roles of each gender.

- The possibility of the existence of two reproductive strategies of males can provide an exciting direction for further investigation to understand the reproductive system of *L. schreiberi*. However, our results are based on a small part of a population. The role of ESS needs confirmation by genetic research and long-term repeated field observations with higher sample sizes to prove the stability of the system and equality of fitness of the two strategies.

- The role of carotenoids as immunostimulant seems not important in *L. schreiberi*, according to the results of the PHA and LPS-induced immune response. However other aspects of the immune system and maybe the natural levels of parasite infections can show the expected relationships. Furthermore, it would be interesting to understand the base of the found link between body condition and carotenoid supplementation or carotenoid-based visual signals.

- The interactions between carotenoids and vitamin E supplementation to visual signals we found were very complicated and very different between signals. Further investigations -maybe in combination of vitamin E with other kind of antioxidants such as vitamin C, or with different doses or different kinds of carotenoids- could present some

interesting interaction effects that could clarify more the relationship of sexual signals and the antioxidant system.

- The life history decisions of immune challenged males of increasing attractiveness in response to survival threats is not unique, however enough rare. Thus, it is possible that there is a back-stair in the reliable signal system because these males would be presenting dishonestly their current condition. This cheating possibility can be a very interesting aspect of the evolution of multiple signals.

- However, we have conceptions about function of multiple signal system of *L. schreiberi*, these have not been supported by experiments yet. In the future, we need to prove that multiple sexual signals of *L. schreiberi* are environmentally influenced, that the preferred signals always mirror an aspect of male quality and that the female mate choice are adaptively changing according to male signal(s) that presumably better indicate the male quality under each given environmental conditions.

- We found that coloration may indicate quality of both females and their offspring. However, color measure before mating was not possible, only after egg laying. Thus, we do not know whether antioxidant investment in coloration change under the vitellogenesis and whether the antioxidant investment into coloration is mobile as can be later invested into eggs. Although we found the base of possible mutual mate choice and we could see that mate guarder males have preferences, other important requirement is to prove that males indeed prefer these color signals to increase their fitness. Furthermore, the origin of different dorsal coloration of females with different female quality traits require more investigations.

References

Aitchison J (1986) The statistical analysis of compositional data. Chapman and Hall, London

Alberts AC (1990) Chemical properties of femoral gland secretions in the desert iguana *Dipsosaurus dorsalis*. J Chem Ecol 16:13–25

Alberts AC (1991) Phylogenetic and adaptive variation in lizard femoral gland secretion. Copeia 1991: 69–79

Alberts AC, Werner DI (1993) Chemical recognition of unfamiliar conspecifics by green iguanas: functional significance of different signal components. Anim Behav 46:197–199

Alberts AC, Pratt NC, Phillips JA (1992) Seasonal productivity of lizard femoral glands: relationship to social dominance and androgen levels. Physiol Behav 51(4): 729–733

Alberts AC, Phillips JA and Werner DI (1993) Sources of intraspecific variability in the protein composition of lizard femoral gland secretions. Copeia 1993:775–781

Alegre J, Hernández A, Purroy FJ (1989) Datos sobre el regimen alimentario del cárabo (*Strix aluco* L.) en la provincial de Leon (no de España). Miscellánia Zoológica 13: 209-211

Alonso-Alvarez C, Doutrelant C, Sorci G (2004) Ultraviolet reflectance affects male-male interactions in the blue tit (*Parus caeruleus ultramarinus*). Behav Ecol 15: 805–809

Amo L, López P, Martín J, Douglas ME (2003) Risk level and thermal costs affect the choice of escape strategy and refuge use in the wall lizard, *Podarcis muralis*. Copeia 2003: 899–905

Amo L, López P, Martín J (2005) Flexibility in antipredatory behavior allows wall lizards to cope with multiple types of predators. Ann Zool Fenn In: Finnish Zoological and Botanical Publishing Board, JSTOR, pp 109–121

Amundsen T (2000) Why are female birds ornamented? Trends Ecol Evol 15: 149–155

Anderson ME (1998) Glutathione: an overview of biosynthesis and modulation. Chem Biol Interact 111–112:1–14.

Aragón P, López P, Martín J (2000) Size-dependent chemosensory responses to familiar and unfamiliar conspecific faecal pellets by the Iberian rock lizard, *Lacerta monticola*. Ethology 106: 1115–1128

Aragón P, López P, Martín J (2001) Chemosensory discrimination of familiar and unfamiliar conspecifics by lizards: implications of field spatial relationships between males. Behav Ecol Sociobiol 50:128–133

Arak A, Enquist M (1995) Conflict, receiver bias and the evolution of signal form. Phil Trans R Soc Lond. B 349:337–344

Ardia DR (2008) Ecoimmunology. In: Davison F, Kaspers B, Schat KA (Eds) Avian immunology, Academic Press, London, pp 421-441

Badyaev AV, Hill GE (2000) Evolution of sexual dichromatism: contribution of carotenoid-versus melanin-based coloration. Biol J Linn Soc 69:153–172

Baeckens S, Huyghe K, Palme R, Van Damme R (2016) Chemical communication in the lacertid lizard *Podarcis muralis*: the functional significance of testosterone. Acta Zoologica

Bagnara JT (1965) Cytology and cytophysiology of non-melanophore pigment cells. Int Rev Cytol 20: 173–205

Bagnara JT, Hadley ME (1973) Chromatophores and color change: the comparative physiology of animal pigmentation. Prenctice-Hall, New Jersey

Bagnara JT, Taylor JD, Hadley ME (1968) The dermal chromatophore unit. J Cell Biol 38:67–79

Bajer K, Molnár O, Török J, Herczeg G (2010) Female European green lizards (*Lacerta viridis*) prefer males with high ultraviolet throat reflectance. Behav Ecol Sociobiol 64:2007–2014

Bajer K, Molnár O, Török J, Herczeg G (2011). Ultraviolet nuptial colour determines fight success in male European green lizards (*Lacerta viridis*). Biol Lett 7:866–868

Bajer K, Molnár O, Török J, Herczeg G (2012) Temperature, but not available energy, affects the expression of a sexually selected ultraviolet (UV) colour trait in male European green lizards. PloS One 7:e34359

Ballen C, Healey M, Wilson M, Tobler M, Wapstra E, Olsson M (2012) Net superoxide levels: steeper increase with activity in cooler female and hotter male lizards. J Exp Biol 215:731–735

Barja G (2007) Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: implications for aging studies. Rejuv Res 10:215–224

Baron M, Davies S, Alexander L, Snellgrove D, Sloman KA (2008) The effect of dietary pigments on the coloration and behaviour of flame-red dwarf gourami, *Colisa lalia*. Anim Behav 75:1041–1051

Bas S (1982) La Comunidad Herpetológica de Caurel: Biogeografía y Ecología. Amphibia-Reptilia 1:1-26

Baumeister FAM, Stachel D, Schuster F, Schmid I, Schaller M, Wolff H, Weiss M, Belohradsky BH (2000) Accelerated phase in partial albinism with immunodeficiency (*Griscelli syndrome*): genetics and stem cell transplantation in a 2-Month-Old Girl. Eur J of Pediatr 159:74–78

Benedetto JP, Ortonne JP, Voulout C (1981) Role of thiol compounds in mammalian melanin pigmentation. I. Reduced and oxidized glutathione. J Invest Dermatol 77:402–405

Bennett ATD, Cuthill IC, Partridge JC, Lunau K (1997) Ultraviolet plumage colors predict mate preferences in starlings. P Natl Acad Sci USA 94:8618–8621

Berglund A, Rosenqvist G (2001) Male pipefish prefer ornamented females. Anim Behav 61:345–350

Biard C, Surai PF, Møller AP (2005) Effects of carotenoid availability during laying on reproduction in the blue tit. Oecologia 144:32–44

Biard C, Surai PF, Møller AP (2006) Carotenoid availability in diet and phenotype of blue and great tit nestlings. J Exp Biol 209:1004–1015

Bitton PP, O'Brien EL, Dawson RD (2007) Plumage brightness and age predict extrapair fertilization success of male tree swallows, *Tachycineta bicolor*. Anim Behav 74:1777–1784

Bleu J, Massot M, Haussy C, Meylan S (2012) Experimental litter size reduction reveals costs of gestation and delayed effects on offspring in a viviparous lizard. Proc R Soc Lond B 279:489–498

Blount JD, Houston DC, Møller AP (2000) Why egg yolk is yellow. Trends Ecol Evol 15:47–49

Blount JD, Surai PF, Nager RG, Houston DC, Møller AP, Trewby ML, Kennedy MW (2002) Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. Proc R Soc Lond B 269:29–36

Bonduriansky R (2001) The evolution of male mate choice in insects: a synthesis of ideas and evidence. Biol Rev 76:305–339

Bonnet X, Naulleau G (1994) A body condition index (BCI) in snakes to study reproduction. C R Acad Sci Ser III Sci Vie 317:34–41

Bonser RHC (1995) Melanin and the abrasion resistance of feathers. Condor 97:590–590

Boulinier T, Staszewski V (2008) Maternal transfer of antibodies: raising immuno-ecology issues. Trends Ecol Evol 23:282–288

Bradley AJ (1987) Stress and mortality in the red-tailed phascogale, *Phascogale calura* (Marsupialia: Dasyuridae). Gen Comp Endocr 67:85–100

Braña F (1982) La reproduccion en los Saurios de Asturias (Reptilia; Squamata): ciclos gonadales, fecundidad y modalidades reproductoras. Rev Biol Univ Oviedo 1:29-50

Braña F (1993) Shifts in body temperature and escape behaviour of female *Podarcis muralis* during Pregnancy. Oikos 1993:216–222

Braña F (1996) Sexual dimorphism in Lacertid lizards: male head increase vs female abdomen increase? Oikos 1996:511–523

Brigelius-Flohe R, Traber MG (1999) Vitamin E: function and metabolism. FASEB J 13:1145–1155

Brito JC, Abreu FBE, Paulo OS, Rosa HD, Crespo EG (1996) Distribution of Schreiber's green lizard (*Lacerta schreiberi*) in Portugal: a predictive model. Herpetol J 6:43–48

Brito JC, Godinho R, Luís C, Paulo OS, Crespo EG (1999) Management strategies for conservation of the lizard *Lacerta schreiberi* in Portugal. Biol Conserv 89:311–319

Britton G (1995) Structure and properties of carotenoids in relation to function. FASEB 9:1551–1558

Brooks R (1996) Melanin as a visual signal amplifier in male guppies. Naturwissenschaften 83:39–41

Brown GP, Shine R (2004) Effects of reproduction on the antipredator tactics of snakes (*Tropidonophis mairii*, Colubridae). Behav Ecol Sociobiol 56:257–262

Buchanan KL, Evans MR, Goldsmith AR (2003) Testosterone, dominance signalling and immunosuppression in the house sparrow, *Passer domesticus*. Behav Ecol Sociobiol 55:50–59

Bulova SJ (1994) Ecological correlates of population and individual variation in antipredator behavior of two species of desert lizards. Copeia 980–992

Burley N (1977) Parental investment, mate choice, and mate quality. P Natl Acad Sci USA 74:3476–3479

Cabido C, Galán P, López P, Martín J (2009) Conspicuousness-dependent antipredatory behavior may counteract coloration differences in Iberian rock lizards. Behav Ecol 20:362–370

Cade TJ (1960) Ecology of the peregrine and gyrfalcon populations in Alaska. University of California Press, Berkeley

Callejo Rey A, Guitian Rivera J, Bas Lopez S, Sanchez Canals JL, Castro Lorenzo A (1979) Primeros datos sobre la dieta de la nutria, Lutra Lutra (L.), en aguas continentales de Galicia. Doñana Acta Vertebr 6:191-202

Candolin U (2003) The use of multiple cues in mate choice. Biol Rev 78:575–595

Candolin U, Reynolds JD (2001) Sexual signaling in the european bitterling: females learn the truth by direct inspection of the resource. Behav Ecol 12:407–411

Catania A, Cutuli M, Garofalo L, Carlin A, Airaghi L, Barcellini W, Lipton JM (2000) The neuropeptide α-MSH in host defense. Ann NY Acad Sci 917:227–231

Chaine AS, Lyon BE (2008). Adaptive plasticity in female mate choice dampens sexual selection on male ornaments in the lark bunting. Science 319:459–462

Chew BP, Park JS (2004) Carotenoid action on the immune response. J Nutr 134:257–261

Civantos E, Forsman A (2000) Determinants of survival in juvenile *Psammodromus algirus* lizards. Oecologia 124:64–72

Civantos E, Salvador A, Veiga JP (1999) Body size and microhabitat affect winter survival of hatchling *Psammodromus algirus* lizards. Copeia 1999:1112–1117

Clevenger AP (1993) Pine marten (*Martes martes* Linné, 1758) comparative feeding ecology in an island and mainland population of Spain. Z Saugetierkd 58:212–212

Cloudsley-Thompson JL (1999) Multiple factors in the evolution of animal coloration. Naturwissenschaften 86:123–132

Clutton-Brock T (2009) Sexual Selection in females. Anim Behav 77:3–11

Coleman JW (2001) Nitric oxide in immunity and inflammation. Int Immunopharmacol 1:1397–1406

Cook PA, Gage MJG (1995) Effects of risks of sperm competition on the numbers of eupyrene and apyrene sperm ejaculated by the moth *Plodia interpunctella* (Lepidoptera: Pyralidae). Behav Ecol Sociobiol 36:261–268

Cooper WE (1997a) Threat factors affecting antipredatory behavior in the broad-headed skink (*Eumeces laticeps*): repeated approach, change in predator path, and predator's field of view. Copeia 1997:613–619

Cooper WE (1997b) Escape by a refuging prey, the broad-headed skink (*Eumeces laticeps*). Can J Zool 75:943–947

Cooper WE (1997c) Factors affecting risk and cost of escape by the broad-headed skink (*Eumeces laticeps*): Predator Speed, Directness of Approach, and Female Presence. Herpetologica 1997:464–474

Cooper WE (1998) Effects of refuge and conspicuousness on escape behavior by the broad-headed skink (*Eumeces laticeps*). Amphibia-Reptilia 19:103–108

Cooper WE (1999) Tradeoffs between courtship, fighting, and antipredatory behavior by a lizard, *Eumeces laticeps*. Behav Ecol Sociobiol 47:54-59

Cooper WE, Blumstein DT (2015) Escaping from predators: an integrative view of escape decisions. Cambridge University Press, Cambridge

Cooper WE, Wilson DS (2007) Sex and social costs of escaping in the striped plateau lizard *Sceloporus virgatus*. Behav Ecol 18:764–768

Commins MA, Goodger BV, Waltisbuhl DJ, Wright IG (1988*) Babesia bovis*: studies of parameters influencing microvascular stasis of infected erythrocytes. Res Vet Sci 44:226–228

Cordero C, Eberhard WG (2003) Female choice of sexually antagonistic male adaptations: a critical review of some current research. J Evol Biol 16:1–6

Cornwallis CK, Birkhead TR (2007) Experimental evidence that female ornamentation increases the acquisition of sperm and signals fecundity. Proc R Soc Lond B 274:583–590

Costantini D, Bruner E, Fanfani A, Dell'Omo G (2007) Male-biased predation of western green lizards by Eurasian kestrels. Naturwissenschaften 94:1015–1020

Costanzo JP, Grenot C, Lee RE (1995) Supercooling, ice inoculation and freeze tolerance in the European common lizard, *Lacerta vivipara*. J Comp Physiol B 165:238–244

Cote J, Meylan S, Clobert J, Voituron Y (2010) Carotenoid-based coloration, oxidative stress and corticosterone in common lizards. J Exp Biol 213:2116–2124

Cuadrado M, Martín J, López P (2001) Camouflage and escape decisions in the common chameleon *Chamaeleo chamaeleon*. Biol J Linn Soc 72:547–554

Cullum AJ (1998) Sexual dimorphism in physiological performance of whiptail lizards (genus Cnemidophorus). Phys Zool 71:541-552

Cuthill IC, Bennett ATD, Partridge JC, Maier EJ (1999) Plumage reflectance and the objective assessment of avian sexual dichromatism. Am Nat 153:183–200

Dale S, Slagsvold T (1996) Mate choice on multiple cues, decision rules and sampling strategies in female Pied Flycatchers. Behaviour 133:903–944

Dantzer R (2001) Cytokine-induced sickness behavior: where do we stand? Brain Behav Immun 15:7–24

Das BS, Thurnham DI, Das DB (1996) Plasma alpha-tocopherol, retinol, and carotenoids in children with falciparum malaria. Am J Clin Nutr 64:94-100

de Bellocq JG, Porcherie A, Moulia C, Morand S (2007) Immunocompetence does not correlate with resistance to helminth parasites in house mouse subspecies and their hybrids. Parasitol Res 100:321–328

Deen CM, Hutchison VH (2001) Effects of lipopolysaccharide and acclimation temperature on induced behavioral fever in juvenile *Iguana iguana*. J Therm Biol 26:55–63

Deger S, Deger Y, Bicek K, Ozdal N, Gul A (2009) Status of lipid peroxidation, antioxidants, and oxidation products of nitric oxide in equine babesiosis: status of antioxidant and oxidant in equine babesiosis. J Equine Vet Sci 29:743-747

DeNardo DF, Sinervo B (1994) Effects of steroid hormone interaction on activity and home-range size of male lizards. Horm Behav 28:273-287

Denton EJ, Land MF (1971) Mechanism of reflexion in silvery layers of fish and cephalopods. Proc R Soc Lond B 178:43–61

Dewsbury DA (1982) Ejaculate cost and male choice. Am Nat 1982: 601–610

Dhabhar FS (2000) Acute stress enhances while chronic stress suppresses skin immunity: the role of stress hormones and leukocyte trafficking. Ann NY Acad Sci 917:876–893

Dhabhar FS, Mcewen BS (1997) Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: A potential role for leukocyte trafficking. Brain Behav Immun 11:286–306

Di Mascio P, Murphy ME, Sies H (1991) Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. Am J Clinic Nutr 53:194–200

Diep SK, Westneat DF (2013) The integration of function and ontogeny in the evolution of status signals. Behaviour 150:1015–1044

Garcia-Dios IS (2006) Dieta del aguililla calzada en el sur de ávila: importancia de los paseriformes. Ardeola 53:39–54

Domínguez JF, Salvador A (1990) Disponibilidad y uso de recursos tróficos por *Lacerta schreiberi* y *Podarcis bocagei* en simpatría en una localidad de la Cordillera Cantábrica, España. Amphibia-Reptilia 11:237-246

Doucet SM, Meadows MG (2009) Iridescence: a functional perspective. J R Soc Interface 6:115–132

Doucet SM, Montgomerie R (2003) Multiple sexual ornaments in satin bowerbirds: ultraviolet plumage and bowers signal different aspects of male quality. Behav Ecol 14:503–509

Doucet SM, Shawkey MD, Hill GE, Montgomerie R (2006) Iridescent plumage in satin bowerbirds: structure, mechanisms and nanostructural predictors of individual variation in colour. J Exp Biol 209 :380–390

Dreiss AN, Roulin A (2010) Age-related change in melanin-based coloration of barn owls (*Tyto alba*): females that become more female-like and males that become more male-like perform better. Biol J Linn Soc 101:689–704

Ducrest AL, Keller L, Roulin A (2008) Pleiotropy in the melanocortin system, coloration and behavioural syndromes. Trends Ecol Evol 23:502–510

Duke-Cohan JS, Gu J, McLaughlin DF, Xu Y, Freeman GJ, Schlossman SF (1998) Attractin (DPPT-L), a member of the CUB family of cell adhesion and guidance proteins, is secreted by activated human T lymphocytes and modulates immune cell interactions. P Natl Acad Sci USA 95:11336–11341

Duke-Cohan JS, Tang W, Schlossman SF (2002) Attractin: a cub-family protease involved in T cell-monocyte/macrophage interactions. *Adv Exp Med Biol* 477:173-185

Eid Y, Ebeid T, Younis H (2006) Vitamin E supplementation reduces dexamethasone-induced oxidative stress in chicken semen. Br Poult Sci 47:350–356

Endler JA (1980). Natural selection on color patterns in *Poecilia reticulata*. Evolution 34:76–91

Endler JA (1984) Natural and sexual selection on color patterns in poeciliid fishes. Environ Biol Fish 9: 173-190

Endler JA (1990) On the measurement and classification of colour in studies of animal colour patterns. Biol J Linn Soc 41:315–352

Endler JA (1991) Interactions between predators and prey. In: Krebs JR, Davies NB (eds) Behavioural ecology: an evolutionary approach, 3rd edn. Blackwell, Oxford, pp 169–196

Endler JA, Basolo AL (1998) Sensory ecology, receiver biases and sexual selection. Trend Ecol Evol 13:415–420

Erdman JW, Bierer TL, Gugger ET (1993) Absorption and transport of carotenoids. Ann NY Acad Sci 691:76–85

Eriksen MS, Haug A, Torjesen PA, Bakken M (2003) Prenatal exposure to corticosterone impairs embryonic development and increases fluctuating asymmetry in chickens (*Gallus gallus domesticus*). Br Poult Sci 44:690–697

Evans RM (1988) The steroid and thyroid hormone receptor superfamily. Science 240:889–895

Evans MR, Martins TLF, Haley M (1994) The asymmetrical cost of tail elongation in red-billed streamertails. Proc R Soc Lond B: Biological Sciences 256:97–103

Evans MR, Goldsmith AR, Norris SRA (2000) The effects of testosterone on antibody production and plumage coloration in male house sparrows (*Passer domesticus*). Behav Ecol Sociobiol 47:156–163

Feingold KR, Grunfeld C (1992) Role of cytokines in inducing hyperlipidemia. Diabetes 41:97–101

Fergusson B, Bradshaw SD, Cannon JR (1985) Hormonal control of femoral gland secretion in the lizard, *Amphibolurus ornatus*. Gen Comp Endocr 57:371–376

Fisher RA (1930) The genetical theory of natural selection: a complete variorum edition. Oxford University Press, Oxford

Fitch HS, Hillis DM (1984) The *Anolis* dewlap: interspecific variability and morphological associations

with habitat. Copeia 1984:315–323

Fitze PS, Tschirren B, Gasparini J, Richner H (2007) Carotenoid-Based Plumage Colors and Immune Function: Is There a Trade-Off for Rare Carotenoids? Am Nat 169:137–144

Fitzpatrick S, Berglund A, Rosenqvist G (1995) ornaments or offspring: costs to reproductive success restrict sexual selection processes. Biol J Linn Soc 55:251–260

Fitzstephens DM, Getty T (2000) Colour, fat and social status in male damselflies, *Calopteryx maculata*. Anim Behav 60:851–855

Fleishman LJ (2000) Signal function, signal efficiency and the evolution of anoline lizard dewlap color. In: Espmark Y, Amundsen T, Rosenqvist G (eds) Animal signals: signalling and signal design in animal communication. Tapir Academic Press, Trondheim, pp 209–236

Fleishman LJ, Loew ER, Leal M (1993) Ultraviolet vision in lizards. Nature 365:397

Folstad I, Karter AJ (1992) Parasites, bright males, and the immunocompetence handicap. Am Nat 1992:603–622

Forsman A, Appelqvist S (1998) Visual predators impose correlational selection on prey color pattern and behavior. Behav Ecol 9:409–413

Forsman A, Shine R (1995) The adaptive significance of colour pattern polymorphism in the Australian scincid lizard *Lampropholis delicata*. Biol J Linn Soci 55:273–291

Fox SF (1983) Fitness, home-range quality, and aggression in Uta stansburiana. In: Huey RB, Pianka ER, Schoener TW (eds) Lizard ecology: studies of a model organism. Harvard University Press, Cambridge, pp 149–168

Freeman-Gallant CR., Schneider RL, Taff CC, Dunn PO, Whittingham LA (2014) Contrasting patterns of selection on the size and coloration of a female plumage ornament in common yellowthroats. J Evol Biol 27:982–991

French SS, DeNardo DF, Moore MC (2007) Trade-Offs between the Reproductive and Immune Systems: Facultative Responses to Resources or Obligate Responses to Reproduction? Am Nat 170:79–89

Fujii R (1993) Cytophysiology of fish chromatophores. Int Rev Cytol 143:191–255

Galán P (1984) Estudio sobre la biometría, folidosis, diseño y coloración de *Lacerta schreiberi*, Bedriaga, 1878 (Sauria, Lacertidae). Alytes 2:25–54

Galán P, Fernández-Arias G (1993) Anfibios e réptiles de galicia. Edicions Xerais de Galicia, Vigo

Galván I, Alonso-Alvarez C (2008) An intracellular antioxidant determines the expression of a melanin-based signal in a bird. PLoS One 3:e3335

Garant D, Sheldon BC, Gustafsson L (2004) Climatic and temporal effects on the expression of secondary sexual characters: genetic and environmental components. Evolution 58:634–644

García-París M, Martín C, Dorda J, Esteban M (1989) Atlas provisional de los anfibios y reptiles de Madrid. Rev Esp Herpetol 3:237–257

Gasparini J, McCoy KD, Haussy C, Tveraa T, Boulinier T (2001) Induced maternal response to the lyme disease spirochaete *Borrelia burgdorferi sensu lato* in a colonial seabird, the kittiwake *Rissa tridactyla*. Proc R Soc Lond B 268:647–650

Geissmann F, Revy P, Brousse N, Lepelletier Y, Folli C, Durandy A, Chambon P, Dy M (2003) Retinoids regulate survival and antigen presentation by immature dendritic cells. J Exp Med 198:623–634

George SB, Lawrence JM, Lawrence AL, Smiley J, Plank L (2001) Carotenoids in the adult diet enhance egg and juvenile production in the sea urchin *Lytechinus variegatus*. Aquaculture 199:353–369

Geremia E, Corsaro C, Bonomo R, Giardinelli R, Pappalardo P, Vanella A, Sichel G (1984) Eumelanins as free radicals trap and superoxide dismutase activities in amphibia. Comp Biochem Phys B 79:67–69

Godin JG, Dugatkin LA (1996) Female mating preference for bold males in the guppy, *Poecilia reticulata*. P Natl Acad Sci USA 93:10262–10267

Gonzalez G, Sorci G, Smith LC, de Lope F (2001) Testosterone and sexual signalling in male house sparrows (*Passer domesticus*). Behav Ecol Sociobiol 50:557–562

Goodwin TW (1986) Metabolism, nutrition, and function of carotenoids. Annu Rev Nutr 6:273–297

Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J (2005) Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. J Androl 26:349–353

Greenberg N (2002) Ethological aspects of stress in a model lizard, *Anolis carolinensis*. Integr Comp Biol 42:526–540

Grenot CJ, Garcin L, Dao J, Hérold J-P, Fahys B, Tséré-Pagès H (2000) How does the European common lizard, *Lacerta vivipara*, survive the cold of winter? Comp Biochem Phys A 127:71–80

Grill CP, Rush VN (2000) Analysing spectral data: comparison and application of two techniques. Biol J Linn Soc 69:121–138

Hadfield JD, Owens IPF (2006) Strong environmental determination of a carotenoid-based plumage trait is not mediated by carotenoid availability. J Evol Biol 19:1104–1114

Haenel GJ, Smith LC, John-Alder HB (2003) Home-range analysis in Sceloporus undulatus. II. A test of spatial relationships and reproductive success. Copeia 2003:113-123.

Halpern M, Martınez-Marcos A (2003) Structure and function of the vomeronasal system: an update. Prog Neurobiol 70:245–318

Halprin K, Okhawara A (1966) Glutathione and human pigmentation. Arch Dermatol 94:355–357

Harris J, Bird DJ (2000) Supernatants from leucocytes treated with melanin-concentrating hormone (MCH) and α-melanocyte stimulating hormone (α-MSH) have a stimulatory effect on rainbow trout (*Oncorhynchus mykiss*) phagocytes in vitro. Vet Immunol Immunop 76:117–124

Hartley RC, Kennedy MW (2004) Are carotenoids a red herring in sexual display? Trends Ecol Evol 19:353–354

Hasson O (1989) Amplifiers and the handicap principle in sexual selection: a different emphasis. Proc R Soc Lond B 235:383–406

Hasson O (1990) The role of amplifiers in sexual selection: an integration of the amplifying and the fisherian mechanisms. Evol Ecol 4:277–289

Hasson O (1997) Towards a general theory of biological signaling. J Theor Biol 185:139–156

Hawley DM, Hallinger KK, Cristol DA (2009) Compromised immune competence in free-living tree swallows exposed to mercury. Ecotoxicology 18:499–503

Hayward LS, Wingfield JC (2004) Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. Gen Comp Endocrinol 135:365–371

Hearing VJ (1993) Unraveling the melanocyte. Am J Hum Genet 52:1

Heatwole H (1968) Relationship of escape behavior and camouflage in Anoline lizards. Copeia 1968:109–113

Hebets EA, Papaj DR (2005) Complex signal function: developing a framework of testable hypotheses. Behav Ecol Sociobiol 57:197–214

Hedrick AV (2000) Crickets with extravagant mating songs compensate for predation risk with extra caution. Proc R Soc Lond B 267:671–675

Hegyi G, Török J, Garamszegi LZ, Rosivall B, Szöllősi E, Hargitai R (2007) Dynamics of multiple sexual signals in relation to climatic conditions. Evol Ecol Res 9:905–920

Heiblum R, Arnon E, Chazan G, Robinzon B, Gvaryahu G, Snapir N (2001) Glucocorticoid administration during incubation: embryo mortality and posthatch growth in chickens. Poult Sci 80:1357–1363

Heinsohn R, Legge S, Endler JA (2005) Extreme reversed sexual dichromatism in a bird without sex role reversal. Science 309:617–619

Henderson LJ, Heidinger BJ, Evans NP, Arnold KE (2013) Ultraviolet crown coloration in female blue tits predicts reproductive success and baseline corticosterone. Behav Ecol 24:1299–1305

Herring PJ (1994) Reflective systems in aquatic animals. Comp Biochem Phys A 109:513–546

Hill GE, Benkman CW (1995) Exceptional response by female red crossbills to dietary carotenoid supplementation. Wilson Bull 107:555–557

Hill GE, Brawner WR (1998) Melanin–based plumage coloration in the house finch is unaffected by coccidial infection. Proc R Soc Lond B 265:1105–1109

Hoelzer GA (1989) The good parent process of sexual selection. Anim Behav 38:1067–1078

Holland B, Rice WR (1998) Perspective: chase-away sexual selection: antagonistic seduction versus resistance. Evolution 1–7

Hong JH, Kim MJ, Park MR, Kwag OG, Lee IS, Byun BH, Lee SC, Lee KB, Rhee SJ (2004) Effects of vitamin E on oxidative stress and membrane fluidity in brain of Streptozotocin-induced diabetic rats. Clin Chim Acta 340:107–115

Hõrak P, Zilmer M, Saks L, Ots I, Karu U, Zilmer K (2006) Antioxidant protection, carotenoids, and the costs of immune challenge in greenfinches. J Exp Biol 209:4329–4338

Hõrak P, Saks L, Zilmer M, Karu U, Zilmer K (2007) Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. Am Nat 170:625–635

Huang Q-H, Hruby VJ, Tatro JB (1999) Role of central melanocortins in endotoxin-induced anorexia. Am J Physiol-Regul Integr Comp Physiol 276:864–871

Huhta E, Rytkönen S, Solonen T (2003) Plumage brightness of prey increases predation risk: an among-species comparison. Ecology 84:1793–1799

Hunt S, Bennett ATD, Cuthill IC, Griffiths R (1998) Blue tits are ultraviolet tits. Proc R Soc Lond B 265:451–455

Husak JF, Macedonia JM, Fox SF, Sauceda RC (2006) Predation cost of conspicuous male coloration in collared lizards (*Crotaphytus Collaris*): an experimental test using clay-covered model lizards. Ethology 112:572–580

Huxley AF (1968) A theoretical treatment of the reflexion of light by multilayer structures. J Exp Biol 48:227–245

Huyghe K, Herrel A, Adriaens D, Tadić Z, Van Damme R (2009) It is all in the head: morphological basis for differences in bite force among colour morphs of the Dalmatian wall lizard. Biol J Linn Soc 96:13–22

Ichiyama T, Sato S, Okada K, Catania A, Lipton JM (2000) The neuroimmunomodulatory peptide α-MSH. Ann NY Acad Sci 917:221–226

Introne W, Boissy RE, Gahl WA (1999) Clinical, molecular, and cell biological aspects of Chediak–Higashi syndrome. Mol Genet Metab 68:283–303

Itzkowitz M, Draud MJ, Barnes JL, Haley M (1998) does it matter that male beaugregory damselfish have a mate preference? Behav Ecol Sociobiol 42:149–155

Izquierdo MS, Fernandez-Palacios H, Tacon AGJ (2001) Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197:25–42

Janeway CA, Travers P, Walport M, Shlomchik M (2001) Immunobiology. The immune system in health and disease, 5th edition. Garland Publishing, New York

Jawor JM, Breitwisch R (2004) Multiple ornaments in male northern cardinals, *Cardinalis cardinalis*, as indicators of condition. Ethology 110:113–126

Jawor JM, Gray N, Beall SM, Breitwisch R (2004) Multiple ornaments correlate with aspects of condition and behaviour in female northern cardinals, *Cardinalis cardinalis*. Anim Behav 67:875–882

Jenssen TA (1977) Evolution of anoline display behavior. Am Zool 17:203–215

Johnsen A, Delhey K, Andersson S, Kempenaers B (2003) Plumage colour in nestling blue tits: sexual dichromatism, condition dependence and genetic effects. Proc R Soc Lond B 270:1263–1270

Johnsen TS, Hengeveld JD, Blank JL, Yasukawa K, Nolan V (1996) epaulet brightness and condition in female red-winged blackbirds. Auk 1996:356–362

Johnson CR (1970) Escape behavior and camouflage in two subspecies of *Sceloporus occidentalis*. Am Midl Nat 280–282

Johnston TA, Wiegand MD, Leggett WC, Pronyk RJ, Dyal SD, Watchorn KE, Kollar S, Casselman JM (2007) Hatching success of walleye embryos in relation to maternal and ova characteristics. Ecol Freshw Fish 16:295–306

Johnstone RA (1996) Multiple displays in animal communication: 'backup signals' and 'multiple messages'. Philos Trans R Soc Lond B Biol Sci 351:329–338

Johnstone RA (1997) The evolution of animal signals. In: Krebs JR, Davies NB (eds) Behavioural ecology: an evolutionary approach, 4th edn. Blackwell, Oxford, pp 155–178

Johnstone RA, Reynolds JD, Deutsch JC (1996) Mutual mate choice and sex differences in choosiness. Evolution 50:1382–1391

Kahn AT, Dolstra T, Jennions MD, Backwell PR (2013) Strategic male courtship effort varies in concert with adaptive shifts in female mating preferences. Behav Ecol 24:906–913

Kalden DH, Scholzen T, Brzoska T, Luger TA (1999) Mechanisms of the antiinflammatory effects of α-MSH: role of transcription factor NF-κB and adhesion molecule expression. Ann NY Acad Sci 885:254–261

Kasraee B, Sorg O, Saurat JH (2003) SP-01 hydrogen peroxide in the presence of cellular antioxidants mediates the first and key step of melanogenesis: a new concept introducing melanin production as a cellular defence mechanism against oxidative stress. Pigment Cell Res 16:571–571

Kasukawa H, Oshima N, Fujii R (1986) Control of chromatophore movements in dermal chromatic units of blue damselfish - II. The motile iridophore. Comp Biochem Physiol C 3:1–7

Kasukawa H, Oshima N, Fujii R (1987) Mechanism of light reflection in blue damselfish motile iridophore. Zool Sci 4:243–257

Kelly RJ, Murphy TG, Tarvin KA, Burness G (2012) Carotenoid-based ornaments of female and male american goldfinches (*Spinus tristis*) show sex-specific correlations with immune function and metabolic rate. Physiol Biochem Zool 85:348–363

Kemp DJ (2008) Female mating biases for bright ultraviolet iridescence in the butterfly *Eurema hecabe* (Pieridae). Behav Ecol 19:1–8

Kemp DJ, Rutowski RL (2007) Condition dependence, quantitative genetics, and the potential signal content of iridescent ultraviolet butterfly coloration. Evolution 61:168–183

Kemp DJ, Vukusic P, Rutowski RL (2006) Stress-mediated covariance between nano-structural architecture and ultraviolet butterfly coloration. Func Ecol 20:282–289

Kennedy MW, Nager RG (2006) The perils and prospects of using phytohaemagglutinin in evolutionary ecology. Trends Ecol Evol 21:653–655

Keskes-Ammar L, Feki-Chakroun N, Rebai T, Sahnoun Z, Ghozzi H, Hammami S, Zghal K, Fki H, Damak J, Bahloul A (2003) Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. Arch Androl 49:83–94

Keyser AJ, Hill GE (1999) Condition–dependent variation in the blue–ultraviolet coloration of a structurally based plumage ornament. Proc R Soc Lond B 266:771–777

Kilgas P, Tilgar V, Külavee R, Saks L, Hõrak P, Mänd R (2010) Antioxidant protection, immune function and growth of nestling great tits *Parus major* in relation to within-brood hierarchy. Comp Biochem Physiol B Biochem Mol Biol 157:288–293

Kingma SA, Szentirmai I, Szekely T, Bokony V, Bleeker M, Liker A, Komdeur J (2008) Sexual selection and the function of a melanin-based plumage ornament in polygamous penduline tits *Remiz pendulinus*. Behav Ecol Sociobiol 62:1277–1288

Kinoshita S, Yoshioka S (2005) Structural colors in nature: the role of regularity and irregularity in the structure. ChemPhysChem 6:1442–1459

Kirk-Smith M, Booth DA, Carroll D, Davies P (1978) Human social attitudes affected by androstenol. Res Commun Psych Psy 3:379-384.

Kirkpatrick M, Ryan MJ (1991) The evolution of mating preferences and the paradox of the lek. Nature 350:33–38

Klasing KC (1998) Comparative avian nutrition. Cab International, Wallingford

Kodric-Brown A, Nicoletto PF (2001) Female choice in the guppy (*Poecilia reticulata*): the interaction between male color and display. Behav Ecol Sociobiol 50:346–351

Kokko H, Monaghan P (2001) Predicting the direction of sexual selection. Ecol Lett 4:159–165

Kokko H, Brooks R, Jennions MD, Morley J (2003) The evolution of mate choice and mating biases. Proc R Soc Lond B 270:653–664

Kopena R, López P, Martín J (2009) Lipophilic compounds from the femoral gland secretions of male Hungarian green lizards, *Lacerta viridis*. Z Naturforsch C 64:434–440

Kopena R, López P, Martín J, Herczeg G (2011) Vitamin E supplementation increases the attractiveness of males' scent for female European green lizards. PLoS One 6:e19410

Kose M, Møller AP (1999) Sexual selection, feather breakage and parasites: the importance of white spots in the tail of the barn swallow (*Hirundo rustica*). Behav Ecol Sociobiol 45:430–436

Kostic D, White WS, Olson JA (1995) Intestinal absorption, serum clearance, and interactions between lutein and beta-carotene when administered to human adults in separate or combined oral doses. Am J Clin Nutr 62:604–610

Kramer DL, Bonenfant M (1997) Direction of predator approach and the decision to flee to a refuge. Anim Behav 54:289-295

Krinsky NI, Yeum KJ (2003) Carotenoid–radical interactions. Biochem Biophys Res Commun 305:754–760

Künzler R, Bakker TCM (2001) Female preferences for single and combined traits in computer animated stickleback males. Behav Ecol 12:681–685

Kuriyama T, Miyaji K, Sugimoto M, Hasegawa M (2006) Ultrastructure of the dermal chromatophores in a lizard (Scincidae: *Plestiodon latiscutatus*) with conspicuous body and tail coloration. Zool Sci 23:793-799

Labra A, Escobar CA, Aguilar PM, Niemeyer HM (2002) Sources of pheromones in the lizard *Liolaemus tenuis*. Rev Chil Hist Nat 75:141–147

Lailvaux SP, Alexander GJ, Whiting MJ (2003) Sex-based differences and similarities in locomotor performance, thermal preferences, and escape behaviour in the lizard *Platysaurus intermedius wilhelmi*. Physiol Biochem Zool 76:511–521

Lande R (1980) Sexual dimorphism, sexual selection, and adaptation in polygenic characters. Evolution 34:292–305

Law JH, Regnier FE (1971) Pheromones. Annu Rev Biochem 40:533–548

Leal M, Fleishman LJ (2004) Differences in visual signal design and detectability between allopatric populations of Anolis lizards. Am Nat 163:26–39

Leibovitz B, Hu ML, Tappel AL (1990) Dietary supplements of vitamin E, β-carotene, coenzyme Q10 and selenium protect tissues against lipid peroxidation in rat tissue slices. J Nutr 120:97–104

Le Poole IC, Mutis T, Van Den Wijngaard RM, Westerhof W, Ottenhoff T, De Vries RR, Das PK (1993a) A novel, antigen-presenting function of melanocytes and its possible relationship to hypopigmentary disorders. J Immunol 151:7284–7292

Le Poole IC, Van den Wijngaard RM, Westerhof W, Verkruisen RP, Dutrieux RP, Dingemans KP, Das PK (1993b) Phagocytosis by normal human melanocytes in vitro. Exp Cell Res 205:388–395

Lee E, Lee H, Kimura J, Sugita S (2010) Feather microstructure of the black-billed magpie (*Pica pica sericea*) and jungle crow (*Corvus macrorhynchos*). J Vet Med Sci 72:1047–1050

Ligon RA, McCartney KL (2016) Biochemical regulation of pigment motility in vertebrate chromatophores: A review of physiological color change mechanisms. Curr Zool 62:237–252

Lim MLM, Li D (2006) Behavioural evidence of UV sensitivity in jumping spiders (Araneae: Salticidae). J Comp Physiol A 192:871–878

Lima SL (1998) Stress and decision-making under the risk of predation: recent developments from behavioral, reproductive, and ecological perspectives. Adv Study Behav 27:215–290

Lima SL, Dill LM (1990) Behavioral decisions made under the risk of predation: a review and prospectus. Can J Zool 68:619–640

Lin SM, Nieves-Puigdoller K, Brown AC, McGraw KJ, Clotfelter ED (2010) Testing the carotenoid trade-off hypothesis in the polychromatic midas cichlid, Amphilophus citrinellus. Physiol Biochem Zool 83:333–342

Lindberg P (1983) Relations between the diet of Fennoscandian Peregrines *Falco peregrinus* and organochlorines and mercury in their eggs and feathers, with a comparison to the gyrfalcon *Falco rusticolus*. Dissertation, University of Göteborg, Göteborg

Lindström L, Ahtiainen JJ, Mappes J, Kotiaho JS, Lyytinen A, Alatalo RV (2006) Negatively condition dependent predation cost of a positively condition dependent sexual signalling. J Evol Biol 19:649–656

Llorente M, Pérez-Mellado V (1988) Alimentación de *Lacerta schreiberi* Bedriaga, 1878 (Sauria: Lacertidae) en el Sistema Central. Rev Esp Herpetol 3:133-141

Loew ER, Fleishman LJ, Foster RG, Provencio I (2002) Visual pigments and oil droplets in diurnal lizards a comparative study of Caribbean anoles. J Exp Biol 205:927–938

López P, Martín J (2001) Pheromonal recognition of females takes precedence over the chromatic cue in male Iberian wall lizards *Podarcis hispanica*. Ethology 107:901–912

López P, Martín J (2006) Lipids in the femoral gland secretions of male Schreiber's green lizards, *Lacerta schreiberi*. Z Naturforsch C 61:763–768

López P, Aragón P, Martín J (1998) Iberian rock lizards (*Lacerta monticola cyreni*) assess conspecific information using composite signals from faecal pellets. Ethology 104:809–820

López P, Aragón P, Martín J (2003) Responses of female lizards, *Lacerta monticola*, to males' chemical cues reflect their mating preference for older males. Behav Ecol Sociobiol 55:73–79

López P, Amo L, Martín J (2006) Reliable signaling by chemical cues of male traits and health state in male lizards, *Lacerta monticola*. J Chem Ecol 32:473–488

Lozano GA (1994) Carotenoids, parasites, and sexual selection. Oikos 1994:309–311

Macedonia JM, James S, Wittle LW, Clark DL (2000) Skin pigments and coloration in the Jamaican radiation of anolis lizards. J Herpetol 34:99–109

Mackintosh JA (2001) The antimicrobial properties of melanocytes, melanosomes and melanin and the evolution of black skin. J Theor Biol 211:101–113

Magnhagen C (1991) Predation risk as a cost of reproduction. Trends Ecol Evol 6:183–186

Majláth I, Majláthová V (2009) Escape behavior of the green lizard (*Lacerta viridis*) in the Slovak karst. Acta Ethol 12:99–103

Marco A (1994) Autoecología y biología reproductora del lagarto verdinegro (*Lacerta schreiberi*, Bedriaga, 1878) en una población de Media Montaña en la Sierra de Béjar (Salamanca). Dissertation, Universidad de Salamanca, Salamanca

Marco A (1995) Edad de adquisición de madurez sexual y variación interanual del tamaño corporal en una población del lagarto *Lacerta schreiberi*. Rev Esp Herp 9:103–111

Marco A (1996) Sedentarismo, áreas de campeo y selección de microhábitats en el lagarto verdinegro *Lacerta schreiberi*. Doñana Acta Vertebrata 23:45–61

Marco A (2015) Lagarto verdinegro - *Lacerta schreiberi*. In: Salvador A, Marco A (eds) Enciclopedia Virtual de los Vertebrados Españoles. Museo Nacional de Ciencias Naturales, Madrid, http://www.vertebradosibericos.org

Marco A, Pérez-Mellado V (1999) Mate guarding, intrasexual competition and mating success in males of the non-territorial lizard *Lacerta schreiberi*. Ethol Ecol Evol 11:279–286

Marco A, Pollo C (1993) Análisis biogeográfico de la distribución del lagarto verdinegro (*Lacerta schreiberi* Bedriaga 1878). Ecologia 7:457–466

Martin LB, Han P, Lewittes J, Kuhlman JR, Klasing KC, Wikelski M (2006) Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct Ecol 20:290–299

Martín J, López P (1990) Amphibians and reptiles as prey of birds in Southwestern Europe. Smithson Herpetol Inf Serv 82:1-43

Martín J, López P (1995) Influence of habitat structure on the escape tactics of the lizard *Psammodromus algirus*. Can J Zool 73:129–132

Martín J, López P (1996) The escape response of juvenile *Psammodromus algirus* lizards. J Comp Psychol 110:187-192

Martín J, López P (1999a) Nuptial coloration and mate guarding affect escape decisions of male lizards *Psammodromus algirus*. Ethology 105:439–447

Martín J, López P (1999b) When to come out from a refuge: risk-sensitive and state-dependent decisions in an Alpine lizard. Behav Ecol 10:487–492

Martín J, López P (2000a) Costs of refuge use affect escape decisions of Iberian rock lizards *Lacerta monticola*. Ethology 106:483–492

Martín J, López P (2000b) Fleeing to unsafe refuges: effects of conspicuousness and refuge safety on the escape decisions of the lizard *Psammodromus algirus*. Can J Zool 78:265–270

Martín J, López P (2006a) Age-related variation in lipophilic chemical compounds from femoral gland secretions of male lizards *Psammodromus algirus*. Biochem Syst Ecol 34:691–697

Martín J, López P (2006b) Vitamin D supplementation increases the attractiveness of males' scent for female Iberian rock lizards. Proc R Soc Lond B 273:2619–2624

Martín J, López P (2007) Scent may signal fighting ability in male Iberian rock lizards. Biol Lett 3:125–127

Martín J, López P (2008) Female sensory bias may allow honest chemical signaling by male Iberian rock lizards. Behav Ecol Sociobiol 62:1927–1934

Martín J, López P (2009) Multiple color signals may reveal multiple messages in male Schreiber's green lizards, *Lacerta schreiberi*. Behav Ecol Sociobiol 63:1743–1755

Martín J, López P (2010a) Condition-dependent pheromone signaling by male rock lizards: more oily scents are more attractive. Chem Senses 35:253-262

Martín J, López P (2010b) Thermal constraints of refuge use by Schreiber's green lizards, *Lacerta schreiberi*. Behaviour 147:275–284

Martín J, López P (2010c) Multimodal sexual signals in male ocellated lizards *Lacerta lepida*: vitamin E in scent and green coloration may signal male quality in different sensory channels. Naturwissenschaften 97:545–553

Martín J, López P (2014) Pheromones and chemical communication in lizards. In: Rheubert JL, Siegel DS, Trauth SE (eds) The reproductive biology and phylogeny of lizards and tuatara, CRC press, Boca Raton, pp 43–75

Martín J, López P (2015) Condition-dependent chemosignals in reproductive behavior of lizards. Horm behav 68:14-24

Martín J, López P, Cooper WE (2003a) Loss of mating opportunities influences refuge use in the Iberian rock lizard, *Lacerta monticola*. Behav Ecol Sociobiol 54:505–510

Martín J, López P, **Douglas ME** (2003b) Changes in the escape responses of the lizard *Acanthodactylus erythrurus* under persistent predatory attacks. Copeia 2003:408–413

Martín J, Civantos E, Amo L, López P (2007a) Chemical ornaments of male lizards *Psammodromus algirus* may reveal their parasite load and health state to females. Behav Ecol Sociobiol 62:173–179

Martín J, Moreira PL, López P (2007b) Status-signalling chemical badges in male Iberian rock lizards. Funct Ecol 21:568–576

Martín J, López P, Gabirot M, Pilz KM (2007c) Effects of testosterone supplementation on chemical signals of male Iberian wall lizards: consequences for female mate choice. Behav Ecol Sociobiol 61:1275–1282

Martín J, Amo L, López P (2008) Parasites and health affect multiple sexual signals in male common wall lizards, *Podarcis muralis*. Naturwissenschaften 95:293–300

Martins EP, Ord TJ, Slaven J, Wright JL, Housworth EA (2006) Individual, sexual, seasonal, and temporal variation in the amount of sagebrush lizard scent marks. J Chem Ecol 32:881–893

Mashaly MM (1991) Effect of exogenous corticosterone on chicken embryonic development. Poult Sci 70:371–374

Mason RT (1992) Reptilian pheromones. In: Gans C, Crews D (eds) Biology of the Reptilia, vol 18. University of Chicago Press, Chicago, pp 114–228

Mateos C, Carranza J (1997) The role of bright plumage in male–male interactions in the ring-necked pheasant. Anim Behav 54:1205–1214

Mäthger LM, Hanlon RT (2007) Malleable skin coloration in cephalopods: selective reflectance, transmission and absorbance of light by chromatophores and iridophores. Cell Tissue Res 329:179–186

Maynard Smith J (1982) Evolution and the theory of games. Cambridge University Press, Cambridge

McDowell LR (2003) Minerals in animal and human nutrition. Elsevier, Amsterdam

McGraw KJ (2008) An update on the honesty of melanin-based color signals in birds. Pigm Cell Melanoma R 21:133–138

McGraw KJ, Hill GE (2000) Differential effects of endoparasitism on the expression of carotenoid- and melanin-based ornamental coloration. Proc R Soc Lond B 267:1525–1531

McGraw KJ, Ardia DR (2005) Sex differences in carotenoid status and immune performance in zebra finches. Evol Ecol Res 7:251–262

McGraw KJ, Klasing KC (2006) Carotenoids, immunity, and integumentary coloration in red junglefowl (*Gallus gallus*). Auk 123:1161–1171

McGraw KJ, Mackillop EA, Dale J, Hauber ME (2002) Different colors reveal different information: how nutritional stress affects the expression of melanin-and structurally based ornamental plumage. J Exp Biol 205:3747–3755

McGraw KJ, Adkins-Regan E, Parker RS (2005) Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. Naturwissenschaften 92:375–380

McGraw KJ, Crino OL, Medina-Jerez W, Nolan PM (2006) Effect of dietary carotenoid supplementation on food intake and immune function in a songbird with no carotenoid coloration. Ethology 112:1209–1216

Megía-Palma R (2016) Molecular characterization of lizard parasites and their influence on colour ornaments. PhD Dissertation, Universidad Complutense de Madrid, Madrid

Merino S, Potti J (1995) High prevalence of hematozoa in nestlings of a passerine species, the pied flycatcher (*Ficedula hypoleuca*). Auk 112:1041-1043

Miwa S, Yamano K (1999) Retinoic acid stimulates development of adult-type chromatophores in the flounder. J Exp Zool 284:317–324

Mohagheghpour N, Waleh N, Garger SJ, Dousman L, Grill LK, Tusé D (2000) Synthetic melanin suppresses production of proinflammatory cytokines. Cell Immunol 199:25–36

Møller AP (1989) Natural and sexual selection on a plumage signal of status and on morphology in house sparrows, *Passer domesticus*. J Evol Biol 2:125–140

Møller AP, Pomiankowski A (1993) Why have birds got multiple sexual ornaments? Behav Ecol Sociobiol 32:167–176

Møller AP, Biard C, Blount JD, Houston DC, Ninni P, Saino N, Surai PF (2000) Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? Avian Poult Biol Rev 11:137–159

Møller AP, Christiansen SS, Mousseau TA (2011) Sexual signals, risk of predation and escape behavior. Behav Ecol 22:800–807

Molnár O, Bajer K, Török J, Herczeg G (2012) Individual quality and nuptial throat colour in male European green lizards. J Zool 287:233–239

Montgomerie R, Hill GE, McGraw KJ (2006) Analyzing colors. In: Hill GE, McGraw KJ (eds) Bird coloration vol 1. Mechanisms and measurements. Harvard University Press, Cambridge, pp 90–147

Moore IT, Jessop TS (2003) Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. Horm Behav 43:39–47

Moore MC, Lindzey J (1992) The physiological basis of sexual behavior in male reptiles. In: Gans C, Crews D (eds) Biology of the Reptilia, vol 18. University of Chicago Press, Chicago, pp 70-113

Moran NA, Jarvik T (2010) Lateral transfer of genes from fungi underlies carotenoid production in aphids. Science 328:624–627

Muller-Schwarze D (2006) Chemical ecology of vertebrates. Cambridge University Press, Cambridge.

Nagaishi H, Oshima N (1989) Neural control of motile activity of light-sensitive iridophores in the neon tetra. Pigm Cell Melanoma R 2:485–492

Nathan C, Shiloh MU (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. P Natl Acad Sci USA 97:8841–8848

Navara KJ, Hill GE (2003) Dietary carotenoid pigments and immune function in a songbird with extensive carotenoid-based plumage coloration. Behav Ecol 14:909–916

Nordeide JT (2002) Do male sticklebacks prefer females with red ornamentation? Can J Zool 80:1344–1349

Nordeide JT, Rudolfsen G, Egeland ES (2006) Ornaments or offspring? Female sticklebacks (*Gasterosteus Aculeatus L.*) trade off carotenoids between spines and eggs. J Evol Biol 19:431–439

Noyes JA, Vukusic P, Hooper IR (2007) Experimental method for reliably establishing the refractive index of buprestid beetle exocuticle. Opt Express 15:4351–4358

Oliphant LW, Hudon J (1993) Pteridines as reflecting pigments and components of reflecting organelles in vertebrates. Pigm Cell Res 6:205–208

Olsson M, Madsen T (1998) Sexual selection and sperm competition in reptiles. In: Birkhead TR, Møller AP (eds) Sperm competition and sexual selection. Academic Press, San Diego, pp 503–578

Olsson M, Wilson M, Isaksson C, Uller T, Mott B (2008) Carotenoid intake does not mediate a relationship between reactive oxygen species and bright colouration: experimental test in a lizard. J Exp Biol 211:1257–1261

Ortega-Rubio A (1991) Trophic partitioning and community organization in a guild of lizards in La Sierra de Guadarrama, Spain. Ekologia 10:19-29

Ortonne JP (2002) Photoprotective properties of skin melanin. Brit J Dermatol 146:7–10

Oshima N, Kasai A (2002) Iridophores involved in generation of skin color in the zebrafish *Brachydanio rerio*. Forma 17:91–101

Owens IPF, Thompson DBA (1994) Sex differences, sex ratios and sex roles. Proc R Soc Lond B 258:93–99

Palozza P (1998) Prooxidant actions of carotenoids in biologic systems. Nutr Rev 56:257–265

Palozza P, Krinsky NI (1992) β-Carotene and α-tocopherol are synergistic antioxidants. Arch Biochem Biophys 297:184–187

Parker GA (1982) Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. J Theor Biol 96:281–294

Parker GA (1983) Mate quality and mating decisions. In: Bateson P (ed) Mate Choice. Cambridge University Press, Cambridge, pp 141:166

Parker GA, Simmons LW (1996) Parental investment and the control of sexual selection: predicting the direction of sexual competition. Proc R S Lond B 263:315–321

Parker GA, Ball MA, Stockley P, Gage MJG (1996) Sperm competition games: individual assessment of sperm competition intensity by group spawners. Proc R S Lond B 263:1291–1297

Parker GA, Ball MA, Stockley P, Gage MJG (1997) Sperm competition games: a prospective analysis of risk assessment. Proc R S Lond B 264:1793–1802

Partridge JC, Cuthill IC (2010) Animal behaviour: ultraviolet fish faces. Curr Biol 20:318–320

Pérez i de Lanuza, GP, Font E (2014) Now you see me, now you don't: iridescence increases the efficacy of lizard chromatic signals. Naturwissenschaften 101:831-837

Pérez-Mellado V (1983) La herpetofauna de Salamanca: un análisis biogeográfico y ecológico. Salamanca Rev Prov Est 9-10: 9-78

Perry G, LeVering K, Girard I, Garland T (2004) Locomotor performance and social dominance in male Anolis cristatellus. Anim Behav 67:37–47

Peters A, Denk AG, Delhey K, Kempenaers B (2004) Carotenoid-based bill colour as an indicator of immunocompetence and sperm performance in male mallards. J Evol Biol 17:1111–1120

Peters A, Delhey K, Johnsen A, Kempenaers B (2007) The condition-dependent development of carotenoid-based and structural plumage in nestling blue tits: males and females differ. Am Nat 169:122–136

Pickering AD, Christie P (1980) Sexual differences in the incidence and severity of ectoparasitic infestation of the brown trout, *Salmo trutta* L. J Fish Biol 16:669–683

Pike TW, Blount JD, Lindström J, Metcalfe NB (2009) Dietary carotenoid availability, sexual signalling and functional fertility in sticklebacks. Biology Letters 6:191–193

Pitnick S, Markow TA (1994) Male gametic strategies: sperm size, testes size, and the allocation of ejaculate among successive mates by the sperm-limited fly *Drosophila pachea* and its relatives. Am Nat 143:785–819

Poston JP, Hasselquist D, Stewart IRK, Westneat DF (2005) Dietary amino acids influence plumage traits and immune responses of male house sparrows, *Passer domesticus*, but not as expected. Animal Behaviour 70:1171–1181

Potti J, Merino S (1996) Decreased levels of blood trypanosome infection correlate with female expression of a male secondary sexual trait: implications for sexual selection. Proc R Soc Lond B 263:1199–1204

Potti J, Canal D, Serrano D (2013) Lifetime fitness and age-related female ornament signalling: evidence for survival and fecundity selection in the pied flycatcher. J Evol Biol 26:1445–1457

Prota G (2012) Melanins and melanogenesis. Academic Press, San Diego

Prum RO (2006) Anatomy, physics, and evolution of avian structural colors. In: Hill GE, McGraw KJ (eds). Bird coloration, vol 1. Mechanisms and measurements. Harvard University Press, Cambridge, pp. 295-355

Qvarnström A, Forsgren E (1998) Should females prefer dominant males? Trends Ecol Evol 13:498–501

Radabaugh DC (1989) Seasonal colour changes and shifting antipredator tactics in darters. J Fish Biol 34:679–685

Reboul E, Thap S, Perrot E, Amiot MJ, Lairon D, Borel P (2007) Effect of the main dietary antioxidants (carotenoids, γ-tocopherol, polyphenols, and vitamin c) on α-tocopherol absorption. Eur J Clin Nutr 61:1167–1173

Regan EA (1981) Hormone specificity, androgen metabolism, and social behavior. Am Zool 21:257–271

Reguera S, Zamora-Camacho FJ, Moreno-Rueda G (2014) The lizard *Psammodromus algirus* (Squamata: Lacertidae) is darker at high altitudes. Biol J Linn Soc 112:132–141

Reid ML, Weatherhead PJ (1990) Mate-choice criteria of Ipswich sparrows: the importance of variability. Anim Behav 40:538–544

Remeš V, Matysioková B (2013) More ornamented females produce higher-quality offspring in a socially monogamous bird: an experimental study in the great tit (*Parus major*). Front Zool 10:1

Rick IP, Bakker TCM (2008) UV wavelengths make female three-spined sticklebacks (*Gasterosteus aculeatus*) more attractive for males. Behav Ecol Sociobiol 62:439–445

Riley PA (1992) Materia melanica: further dark thoughts. Pigm Cell Res 5:101–106

Rödder D, Schulte U (2010) Potential loss of genetic variability despite well established network of reserves: the case of the Iberian endemic lizard *Lacerta schreiberi*. Biodivers Conserv 19:2651–2666

Rodgers GM, Gladman NW, Corless HF, Morrell LJ (2013) Costs of colour change in fish: food intake and behavioural decisions. J Exp Biol 216:2760–2767

Rohr JR, Madison DM (2001) A chemically mediated trade-off between predation risk and mate search in newts. Anim Behav 62:863–869

Romero LM (2002) Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. Gen Comp Endocrinol 128:1–24

Roulin A (2015) Condition-dependence, pleiotropy and the handicap principle of sexual selection in melanin-based colouration. Biol Rev 91:328-48

Roulin A, Richner H, Ducrest AL (1998) Genetic, environmental, and condition-dependent effects on female and male ornamentation in the barn owl *Tyto alba*. Evolution 52:1451–1460

Roulin A, Riols C, Dijkstra C, Ducrest AL (2001) Female plumage spottiness signals parasite resistance in the barn owl (*Tyto alba*). Behav Ecol 12:103–110

Roulin A, Almasi B, Rossi-Pedruzzi A, Ducrest AL, Wakamatsu K, Miksik I, Blount JD, Jenni-Eiermann S, Jenni L (2008) Corticosterone mediates the condition-dependent component of melanin-based coloration. Anim Behav 75:1351–1358

Roulin A, Altwegg R, Jensen H, Steinsland I, Schaub M (2010) Sex-dependent selection on an autosomal melanic female ornament promotes the evolution of sex ratio bias. Ecol Lett 13:616–626

Różanowska M, Sarna T, Land EJ, Truscott TG (1999) Free radical scavenging properties of melanin: interaction of eu-and pheo-melanin models with reducing and oxidising radicals. Free Radical Bio Med 26:518–525

Rubolini D, Romano M, Boncoraglio G, Ferrari RP, Martinelli R, Galeotti P, Fasola M, Saino N (2005) Effects of elevated egg corticosterone levels on behavior, growth, and immunity of yellow-legged gull (*Larus michahellis*) chicks. Horm Behav 47:592–605

Ruiz M, Wang D, Reinke BA, Demas GE, Martins EP (2011) Trade-offs between reproductive coloration and innate immunity in a natural population of female sagebrush lizards, *Sceloporus graciosus*. Herpetol J 21:131

Ruiz-de-Castañeda R, Burtt EH, González-Braojos S, Moreno J (2012) Bacterial degradability of an intrafeather unmelanized ornament: a role for feather-degrading bacteria in sexual selection? Biol J Linn Soc 105:409–419

Ryan MJ, Fox JH, Wilczynski W, Rand AS (1990) Sexual selection for sensory exploitation in the frog *Physalaemus pustulosus*. Nature 343:66-7

Paulo Sá-Sousa, Rafael Marquez, Valentin Pérez-Mellado, Iñigo Martínez-Solano (2009) *Lacerta schreiberi*. The IUCN Red List of Threatened Species 2009: eT11113A3251580

Saenko SV, Teyssier J, Van Der Marel D, Milinkovitch MC (2013) Precise colocalization of interacting structural and pigmentary elements generates extensive color pattern variation in *Phelsuma* lizards. BMC biol 11:105.

Salaberria C, Muriel J, de Luna M, Gil D, Puerta M (2013) The PHA test as an indicator of phagocytic activity in a passerine bird. PloS One 8:e84108

Saleh MA (2009) Erythrocytic oxidative damage in crossbred cattle naturally infected with *Babesia bigemina*. Res Vet Sci 86:43–48

San-José LM, Granado-Lorencio F, Sinervo B, Fitze PS (2013) Iridophores and not carotenoids account for chromatic variation of carotenoid-based coloration in common lizards (*Lacerta vivipara*). Am Nat 181:396–409

Santos ESA, Scheck D, Nakagawa S (2011) Dominance and plumage traits: meta-analysis and metaregression analysis. Anim Behav 82:3–19

Sarv T, Hõrak P (2009) Phytohaemagglutinin injection has a long-lasting effect on immune cells. J Avian Biol 40:569–571

Sasaki M, Horikoshi T, Uchiwa H, Miyachi Y (2000) Up-regulation of tyrosinase gene by nitric oxide in human melanocytes. Pigm Cell Res 13:248–252

Sassoon DA, Gray GE, Kelley DB (1987) Androgen regulation of muscle fiber type in the sexually dimorphic larynx of *Xenopus laevis*. J Neurosci 7:3198–3206

Savalli UM, Fox CW (1999) The effect of male mating history on paternal investment, fecundity and female remating in the seed beetle *Callosobruchus Maculatus*. Funct Ecol 13:169–177

Scheuerlein A, Van't Hof T, Gwinner E (2001) Predators as stressors? Physiological and reproductive consequences of predation risk in tropical stonechats (*Saxicola torquata axillaris*). Proc R Soc Lond B 268:1575–1582

Schiedt K (1998) Absorption and metabolism of carotenoids in birds, fish and crustaceans. Carotenoids 3:285-358

Schwabl H (1996) Maternal testosterone in the avian egg enhances postnatal growth. Comp Biochem Phys A 114:271–276

Schwenk K (1995) Of tongues and noses: chemoreception in lizards and snakes. Trends in Ecology & Evolution 10:7–12

Seagle BLL, Rezai KA, Kobori Y, Gasyna EM, Rezaei KA, Norris JR (2005) Melanin photoprotection in the human retinal pigment epithelium and its correlation with light-induced cell apoptosis. P Natl Acad Sci USA 102:8978–8983

Secondi J, Lepetz V, Théry M (2012) Male attractiveness is influenced by uv wavelengths in a newt species but not in its close relative. PLoS One 7:e30391

Shashar N, Rutledge P, Cronin T (1996) Polarization vision in cuttlefish in a concealed communication channel? J Exp Biol 199:2077–2084

Shcherba VV, Babitskaya VG, Kurchenko VP, Ikonnikova NV, Kukulyanskaya TA (2000) Antioxidant properties of fungal melanin pigments. Appl Biochem Micro+ 36:491–495

Siebeck UE (2004) Communication in coral reef fish: the role of ultraviolet colour patterns in damselfish territorial behaviour. Anim Behav 68:273–282

Siefferman L, Hill GE (2005) Evidence for sexual selection on structural plumage coloration in female eastern bluebirds (*Sialia sialis*). Evolution 59:1819–1828

Silverin B (1980) Effects of long-acting testosterone treatment on freeliving pied flycatchers, *Ficedula hypoleuca*, during the breeding period. Anim Behav 28:906–912

Silverin B (1998) Behavioural and hormonal responses of the pied flycatcher to environmental stressors. Anim Behav 55:1411–1420

Simonis P, Berthier S (2012) How nature produces blue color. In: Massaro A (ed) Photonic Crystals – Introduction, applications and theory. InTech, Rijeka

Simonis P, Vigneron JP (2011) Structural color produced by a three-dimensional photonic polycrystal in the scales of a longhorn beetle: *Pseudomyagrus waterhousei* (Coleoptera: Cerambicidae). Phys Rev E 83:11908

Skarstein F, Folstad I (1996) Sexual dichromatism and the immunocompetence handicap: an observational approach using arctic charr. Oikos 76:359–367

Smit N, Le Poole I, van den Wijngaard R, Tigges A, Westerhof W, Das P (1993) Expression of different immunological markers by cultured human melanocytes. Arch Dermatol Res 285:356–365

Smith DG (1996) Correlates of approach distance in the striped plateau lizard (*Sceloporus virgatus*). Herpetol J 6:56–58

Smith DG (1997) Ecological factors influencing the antipredator behaviors of the ground skink, *Scincella lateralis*. Behav Ecol 8:622–629

Smits JE, Bortolotti GR, Tella JL (1999) Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. Funct Ecol 13:567–572

Solano F, García-Borrón JC (2006) Enzymology of melanin formation. In: Nordlund JJ, Boissy RE, Hearing VJ, King RA, Oetting WS, Ortonne JP (eds) The pigmentary system: physiology and pathophysiology, 2nd edn. Blackwell, Oxford, pp 261–281

Speake BK, Surai PF, Gore M (2001) Lipid composition, fatty acid profiles, and lipid-soluble antioxidants of eggs of the Hermann's tortoise (*Testudo hermanni boettgeri*). Zoo Biol 20:75–87

Spritz RA (2000) Hermansky–Pudlak syndrome and pale ear: melanosome-making for the millennium. Pigm Cell Res 13:15–20

Squires EL, Pickett BW, Amann RP (1978) Effect of successive ejaculation on stallion seminal characteristics. J Rep Fer S 27:7–12

Stankowich T, Blumstein DT (2005) Fear in animals: a review and meta-analysis of risk assessment. Proc R Soc Lond B 272:2627-2634

Stapley J, Whiting MJ (2006) Ultraviolet signals fighting ability in a lizard. Biol Lett 2:169–172

Steffen JE, Hill GE, Guyer C (2010) Carotenoid access, nutritional stress, and the dewlap color of male brown anoles. Copeia 2010:239–246

Stuart-Fox DM, Moussalli A, Marshall NJ, Owens IPF (2003) Conspicuous males suffer higher predation risk: visual modelling and experimental evidence from lizards. Anim Behav 66:541–550

Sugumaran M (2002) Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigm Cell Res 15:2–9

Suleiman SA, Elamin Ali M, Zaki ZMS, El-Malik EMA, Nasr MA (1996) Lipid peroxidation and human sperm motility: protective role of vitamin E. J Androl 17:530–537

Surai PF (2002) Natural antioxidants in avian nutrition and reproduction. Nottingham University Press, Nottingham

Surai PF, Kutz E, Wishart GJ, Noble RC, Speake BK (1997) The relationship between the dietary provision of α-tocopherol and the concentration of this vitamin in the semen of chicken: effects on lipid composition and susceptibility to peroxidation. J Reprod Fertil 110:47–51

Surai PF, Speake BK, Sparks NHC (2001a) Carotenoids in avian nutrition and embryonic development. 2. Antioxident properties and discrimination in embryonic tissues. J Poult Sci 38:117–145

Surai PF, Speake BK, Sparks NHC (2001b) Carotenoids in avian nutrition and embryonic development. 1. Absorption, availability and levels in plasma and egg yolk. J Poult Sci 38:1–27

Sutherland JL, Thompson CF, Sakaluk SK (2012) No effect of carotenoid supplementation on PHA response or body condition of nestling house wrens. Physiol Biochem Zool 85:21–28

Számadó S (2000) Cheating as a mixed strategy in a simple model of aggressive communication. Anim Behav 59: 221-230

Szigeti B, Török J, Hegyi G, Rosivall B, Hargitai R, Szõllõsi E, Michl G (2007) Egg quality and parental ornamentation in the blue tit *Parus caeruleus*. J Avian Biol 38:105–112

Takeuchi DIK (1976) Electron microscopy of two types of reflecting chromatophores (iridophores and leucophores) in the guppy, *Lebistes reticulatus* Peters. Cell Tissue Res 173:17–27

Teyssier J, Saenko SV, Van Der Marel D, Milinkovitch MC (2015) Photonic crystals cause active colour change in chameleons. Nat Commun 6:6368

Thompson MB, Speake BK (2002) Energy and nutrient utilisation by embryonic reptiles. Comp Biochem Phys A 133:529–538

Thusius KJ, Peterson KA, Dunn PO, Whittingham LA (2001) Male mask size is correlated with mating success in the common yellowthroat. Anim Behav 62:435–446

Tobler M, Healey M, Wilson M, Olsson M (2011) Basal superoxide as a sex-specific immune constraint. Biol Lett 7:906–908

Tokarz RR (1995) Mate choice in lizards: a review. Herpetol Monogr 8:17–40

Tomás G, Merino S, Martínez J, Moreno J, Sanz JJ (2005). Stress protein levels and blood parasite infection in blue tits (*Parus caeruleus*): a medication field experiment. Ann Zool Fenn 42:45–56

Toomey MB, Butler MW, McGraw KJ (2010) Immune-system activation depletes retinal carotenoids in house finches (*Carpodacus mexicanus*). J Exp Biol 213:1709–1716

Tsatmali M, Graham A, Szatkowski D, Ancans J, Manning P, McNeil CJ, Graham AM, Thody AJ (2000) α-Melanocyte-stimulating hormone modulates nitric oxide production in melanocytes. J Invest Dermatol 114:520–526

Tyssandier V, Cardinault N, Caris-Veyrat C, Amiot MJ, Grolier P, Bouteloup C, Azais-Braesco V, Borel P (2002) Vegetable-borne lutein, lycopene, and β-carotene compete for incorporation into chylomicrons, with no adverse effect on the medium-term (3-wk) plasma status of carotenoids in humans. Am J Clin Nutr 75:526–534

Tyssandier V, Reboul E, Dumas JF, Bouteloup-Demange C, Armand M, Marcand J, Sallas M, Borel P (2003) Processing of vegetable-borne carotenoids in the human stomach and duodenum. Am J Physiol-Gastr L 284:913–923

Uller T, Isaksson C, Olsson M (2006) Immune challenge reduces reproductive output and growth in a lizard. Funct Ecol 20:873–879

Verwaijen D, Van Damme R (2008) Foraging mode and its flexibility in lacertid lizards from Europe. J Herpetol 42:124–133

Vigneron JP, Colomer JF, Rassart M, Ingram AL, Lousse V (2006) Structural origin of the colored reflections from the black-billed magpie feathers. Phys Rev E 73:21914

Vinkler M, Bainová H, Albrecht T (2010) Functional analysis of the skin-swelling response to phytohaemagglutinin. Funct Ecol 24:1081–1086

Vinkler M, Schnitzer J, Munclinger P, Albrecht T (2012) Phytohaemagglutinin skin-swelling test in scarlet rosefinch males: low-quality birds respond more strongly. Anim Behav 83:17–23

Vitt LJ, Cooper WE (1985) The evolution of sexual dimorphism in the skink *Eumeces laticeps*: an example of sexual selection. Can J Zool 63:995–1002

von Schantz T, Bensch S, Grahn M, Hasselquist D, Wittzell H (1999) Good genes, oxidative stress and condition–dependent sexual signals. Proc R Soc Lond B 266:1–12

Wedekind C (1992) Detailed information about parasites revealed by sexual ornamentation. Proc R Soc Lond B 247:169–174

Weiss SL (2006) Female-specific color is a signal of quality in the striped plateau lizard (*Sceloporus virgatus*). Behav Ecol 17:726–732

Weiss SL, Kennedy EA, Bernhard JA (2009) Female-specific ornamentation predicts offspring quality in the striped plateau lizard, *Sceloporus virgatus*. Behavioral Ecology 20:1063-1071

Weiss SL, Kennedy EA, Safran RJ, McGraw KJ (2011) Pterin-based ornamental coloration predicts yolk

Weiss SL, Mulligan EE, Wilson DS, Kabelik D (2013) Effect of stress on female-specific ornamentation. J Exp Biol 216:2641–2647antioxidant levels in female striped plateau lizards (*Sceloporus virgatus*). J Anim Ecol 80:519–527

Weldon PJ, Flachsbarth B, Schulz S (2008) Natural products from the integument of nonavian reptiles. Nat Prod Rep 25:738–756

Whiting MJ (2002) Field experiments on intersexual differences in predation risk and escape behaviour in the lizard *Platysaurus broadleyi*. Amphibia-Reptilia 23:119–124

Whiting MJ, Stuart-Fox DM, O'Connor D, Firth D, Bennett NC, Blomberg SP (2006) Ultraviolet signals ultra-aggression in a lizard. Anim Behav 72:353–363

Wiegand MD (1996) Composition, accumulation and utilization of yolk lipids in teleost fish. Rev Fish Biol Fisher 6:259–286

Wingfield JC, Ball GF, Dufty AM, Hegner RE, Ramenofsky M (1987) Testosterone and aggression in birds. Am Sci 75:602–608

Woodall AA, Britton G, Jackson MJ (1996) Dietary supplementation with carotenoids: effects on α-tocopherol levels and susceptibility of tissues to oxidative stress. Br J Nutr 76:307–317

Wu D, Meydani SN (1998) N-3 polyunsaturated fatty acids and immune function. P Nutr Soc 57:503–509

Wu G, Fang YZ, Yang S, Lupton JR, Turner ND (2004) Glutathione metabolism and its implications for health. J Nutr 134:489–492

Wyatt TD (2003) Pheromones and animal behaviour: communication by smell and taste. Cambridge University Press, Cambridge

Yoshida M, Takahashi Y, Inoue S (2000) Histamine induces melanogenesis and morphologic changes by protein kinase A activation via H2 receptors in human normal melanocytes. J Invest Dermatol 114:334–342

Zahavi A (1975) Mate selection—a selection for a handicap. J Theor Biol 53:205–214

Zi J, Yu X, Li Y, Hu X, Xu C, Wang X, Liu X, Fu R (2003) Coloration strategies in peacock feathers. P Natl Acad Sci USA 100:12576–12578

Chapter I

This chapter reproduces entirely the manuscript:

Kopena R, Herczeg G, López P, Martín J (2015) Escape strategy of Schreiber's green lizard (*Lacerta schreiberi*) is determined by the environment but not season or sex. Behaviour 152:1527-1542

Escape strategy of Schreiber's green lizards (*Lacerta schreiberi*) is determined by environment but not season or sex

Renáta Kopena [a], Gábor Herczeg [b], Pilar López [a] and José Martín [a]

[a] *Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas, José Gutiérrez Abascal 2, E-28006 Madrid, Spain*

[b] *Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/c, H-1117, Budapest, Hungary*

Abstract

Antipredator escape behaviour varies with several well-established sources of variation ranging from the physical environment to reproductive status. However, the relative roles of these sources are rarely assessed together. We measured (i) the distance to the nearest refuge that Schreiber's green lizards, *Lacerta schreiberi*, maintained before an attack (refuge distance) and (ii) the distance lizards allowed a simulated predator to approach before fleeing (flight initiation distance, FID). Refuge distance was unaffected by studied variables. However, FID was positively related to refuge distance on grassy, but not on rocky substrates. Furthermore, refuge distance and escape angle interacted in a substrate-independent manner: lizards allowed predators close when refuges were close or when lizards had to flee towards the predator. In contrast, neither mating season nor sex affected FID. We suggest that the escape strategy of *L. schreiberi* is determined more by the physical environment than by sex or reproductive condition.

Keywords: antipredator behavior · refuge distance · escape angle · escape behavior · flight initiation distance · Squamata.

Introduction

Predation is one of the most important selection pressures that shapes the morphology (Endler 1991) and behaviour of animals (Lima 1998). This is because avoiding or surviving predatory attacks is key for increasing fitness (Lima and Dill 1990). However, escape behaviour can be costly too, because by moving the prey may be more conspicuous for the predator, wasting energy and foraging opportunities. Consequently, animals are predicted to escape when the fitness costs of remaining stationary equal or exceed the costs of escaping (Ydenberg and Dill 1986) or when the prey's expected fitness after the encounter is maximized (Cooper and Frederick 2007).

Given the variation in the actual cost-benefit relationships, antipredator behaviour must be, and is, very flexible. For instance, the effect of habitat structure on escape decisions is very important (Snell et al. 1988; Martín and López 1995; Majláth and Majláthova 2009). In dense vegetation, the flight initiation distance (FID, the distance between predator and prey when prey starts to flee) is generally shorter than in open (e.g., rocky) habitat, because the prey can use cryptic behaviour in the former, while it is more conspicuous in the latter (Martín and López 1995; Snell et al. 1988; Majláth and Majláthova 2009; but see Smith 1997).

Another relevant environmental factor is the relative position of the predator, the prey and the prey's refuge. How close an animal allows a predator to approach is strongly dependent on the distance and direction to the nearest available refuge. In many studies FID and distance to the nearest refuge are positively correlated (Dill and Houtman 1989; Cooper 1997a; Stankovich and Blumstein 2005; but see Cooper and Wilson 2007). The direction of escape with respect to the predator position is one of the most important variables of escape success (Domenici et al. 2011a, b). This escape direction depends on many factors such as the availability and position of refuge(s) and obstacles in the way, or the presence of conspecifics or another predator (Domenici et al. 2011a, b). If there is a refuge, the safest escape direction is directly towards the refuge (Cooper 1997a) or following a direction that

maximizes the distance from the predator while minimizing the time needed to reach the refuge (Zani et al. 2009; Domenici et al. 2011a, b). However, if the animal is too far from a refuge, instead of running directly to hide in the refuge, the animal may change its escape behaviour, running in circles or with many changes in escape direction, without using the refuge (Zani et al. 2009). The escape direction can be characterized by the escape angle (i.e., the angle formed by (i) the line connecting the prey and the approaching predator and (ii) the escape path of the prey; see Methods). This variable is overlooked in many studies, but it is very important, especially in prey species that have a permanent refuge (Domenici et al. 2011b). The FID increases with refuge distance and escape angle in broad-headed skinks, *Eumeces laticeps* (Cooper 1997a), and in eastern woodchucks, *Marmota monax* (Kramer and Bonenfant 1997).

In addition to the effects of vegetative cover, distance to refuge, and escape angle on FID, many other factors affect escape decisions (Stankowich and Blumstein 2005; Cooper and Frederick 2007). For example, relative conspicuity to predators of different individuals may affect the risk of being detected and this should affect escape decisions. Visual conspicuousness of coloration correlates positively with shyness in many animals (Forsman and Appelqvist 1998; Martín and López 1999a; Cuadrado et al. 2001; Lindström et al. 2007; Cabido et al. 2009; Møller et al. 2011; but see Godin and Dugatkin 1996). In some lizards, FID varies among species, apparently being inversely correlated with the degree of cryptic coloration (Heatwole 1968; Johnson 1970; however, these findings are highly questionable due to statistical inadequacies and lack of phylogenetic perspective), or within a species as a function of the degree of conspicuousness in different microhabitats (Martín and López 1995, 2000a; Cooper 1998a; Cuadrado et al. 2001).

In some species, there are also differences between the escape tactics of males and females. For example, in lizards with sexually dichromatic coloration, males, with more conspicuous coloration, have longer FIDs than females in some species (Martín and López 1999a; Lailvaux et al. 2003) but not in others (Smith 1996, 1997; Whiting 2002; Cooper and Wilson 2007). Male green lizards, *Lacerta viridis*, have longer FIDs than females before and after the mating season, but during the mating season the difference dissipates (Majláth and

Majláthová 2009).

In the present study, we examined the relative roles of microhabitat, position of the threat and refuge, season, and sex in determining the escape strategy of adult Schreiber's green lizards, *Lacerta schreiberi*, a large lacertid lizard with strong sexual dichromatism (Martín and López 2009; Salvador 2014). We considered two parts of the antipredatory behaviour of lizards: a preventive risk-taking behaviour (i.e., the overall distance to the nearest potential refuge that the lizard maintains before an attack) (e.g., Carrascal et al. 1992) and an active escape decision in an emergency situation when an attack occurs (i.e., the distance to the predator when a lizard starts fleeing) (Cooper and Frederick 2007). We predicted that lizards should maintain closer distances to the refuge when they perceive a potentially higher risk, such as in open microhabitats or when the lizard is more conspicuous. We also predicted that FID increases as distance to refuge increases and as the escape angle relative to the predator increases, where 0° represents fleeing directly away from the predator. We further predicted that these effects would be less pronounced in exposed (i.e., open rocks) than in complex (grassy areas) microhabitats because the detectability of lizards by the predator and the attack success should be higher on open areas than in dense vegetation. We also assessed differences in antipredatory behaviour between mating and non-mating seasons and between sexes. We expected males having shorter distances to refuge and longer FIDs than females in any season, because they are more conspicuous due to their brighter coloration (Martín and López 2009), while both males and females should accept greater risk in the mating than in the non-mating season to avoid losing reproductive opportunities (Magnhagen 1991; Cooper 1999b; Martín et al. 2003). To test the above predictions, we conducted a field study of the escape behaviour of adult lizards of both sexes in different microhabitats and both within and outside of the mating season.

Materials and methods

Data collection

The study was performed during the summer of 2008 and spring of 2009 in a large pine forest ('Valle de La Fuenfría') in the Guadarrama mountains (Madrid Province, Spain; 40°44´N, 4°02´W). The dominant vegetation consists of *Pinus sylvestris* forest, with shrubs such as *Juniperus communis* and *Cytisus scoparius*. In this area, Schreiber's green lizards are active from March to September, mate in April–May, and produce a single clutch during June (Marco and Pérez-Mellado 1999). Lizards occupy relatively moist well vegetated areas, often close to streams (Salvador, 1988, 2014). In this area the most frequent predators of *L. schreiberi* are common kestrels (*Falco tinnunculus*), common buzzards (*Buteo buteo*), booted eagles (*Hieraaetus pennatus*), grass snakes (*Natrix natrix*), cats (*Felis catus*), dogs (*Canis familiaris*) and foxes (*Vulpes vulpes*).

We observed the escape behaviour of 31 adult females and 12 males in August 2008 and 36 males and 22 females in May 2009. Because the spring sample (May) coincided with the first part of the mating season, females were at most in early stages of gravidity; in summer (August) reproduction had finished and all females had already laid their eggs. This means that lizard escape behaviour was assessed during and after the mating season, respectively. However, the samples were collected in different years, hence, year effects stemming from several potential sources (different hibernation survival, sex ratios, climatic conditions, food supply, predator/parasite densities, etc.) cannot be entirely ruled out, potentially hampering our ability to make clear conclusions on the role of reproductive state in escape decisions. We searched for lizards between 10.00 and 18.00 h. The observations were carried out in sunny, warm and unwindy weather. One of us (RK) wearing the same clothing simulated an approaching predator. This is a conventional method in studies of lizard escape behaviour, because lizards identify humans as natural predators (e.g., Braña 1993; Bulova 1994; Martín and López 1995, 1999a, 2000a, b, 2003; Cooper 1997a, b, c, 1998a, b; Amo et al. 2003, 2005).

The observer walked at approx. 1 m/s until an adult lizard was located. After this, the observer simulated a predatory attack by approaching the lizard directly at a slow speed (approx. 0.6 m/s) until the lizard fled. We recorded the sex of animals (sexual dichromatism was easily discerned by sight) and the following behavioral variables: (i) 'refuge distance', the distance between the lizard and the refuge it later used to hide, (ii) the flight initiation distance ('FID'), the distance between the observer and the lizard when the lizard started fleeing and (iii) 'escape angle', which was the angle formed by the line connecting the initial position of the lizard and the observer and the line connecting the lizard and the refuge or first stopping point after escaping (Martín and López 1996; Cooper 1997a). An escape angle of 0° indicated the escape direction was directly away from the predator, while 180° meant the lizards escaped directly towards the approaching predator (Martín and López 1996). The orientation of escape direction was not considered because it was immaterial whether movements were to right or left, as it presumably does not affect the probability of escape (Cooper 1997a). We also noted the microhabitat in which the lizard was initially located (grass vs. open rocks). The observations were made in different parts of a natural large field area where lizard density was high. Therefore, we assumed that the chance of repeated observations of the same individuals was low, and considered the data points to be independent (Bulova 1994; Martín and López 1995; Cooper 1997b).

Statistical analyses

We ran two General Linear Models (GLMs). The first GLM was built with refuge distance as the dependent variable, and sex, microhabitat type and season as fixed factors to reveal patterns related to general risk-taking. Second, we ran a GLM with FID as the dependent variable, sex, microhabitat type and season as fixed factors and refuge distance and escape angle as covariates to reveal patterns of escape strategy. Refuge distance and escape angle did not correlate ($r = -0.08$; $N = 101$; $p = 0.42$), hence collinearity between the covariates can be ruled out. The original models included all single effects and two-way interactions. We note that our second model might be over-parametrized based on the sample size/number of effects in the model ratio, but the simple fact that model selection (see below)

did not alter the results (i.e., the final model including only five effects showed the same pattern as the full model) makes overparametrization as a significant bias unlikely. We applied backward stepwise model selection based on the $p < 0.05$ criterion. There are several model selection approaches available, but this one is generally considered as a conservative choice (Murtaugh 2009). We first removed the nonsignificant interactions in the order of decreasing p value and then did the same with the single effects. We never removed single effects that were part of significant interactions. We also checked whether the distribution of model residuals deviated from normality by visually inspecting histograms and Q–Q plots. There was a trend towards positive skewness in the first GLM (on refuge distance). After logarithmic ($\log_{10}$) transformation, the residuals distributed normally, but the transformation did not affect the GLM results, hence we report the results from the original model. All analyses were done by using the SPSS 17.0.1 (SPSS, Chicago, IL, USA) statistical software.

Results

Refuge distance was not significantly related to any of the predictor variables (Table 1). However, FID was significantly affected by microhabitat, refuge distance, their interaction and the refuge distance × escape angle interaction, but not by sex, season or any of their interactions (Table 2). Backward stepwise selection produced results similar to those of the original model for all variables. Effect sizes for the microhabitat × refuge distance interaction and the single microhabitat effects can be interpreted as large, while for the refuge distance × escape angle interaction and the single effect of refuge distance as small-medium (e.g. Cohen 1992).

FID was positively related to refuge distance in the grass microhabitat ($R^2 = 0.23$, $p < 0.001$; Figure 1a), but not in the rocky microhabitat ($R^2 = 0.06$, $p = 0.22$; Figure 1b). The refuge distance × escape angle interaction (Figure 2) occurred because (1) if a lizard was close to the refuge, it allowed the predator to approach closer independently of escape angle, (2) if the escape angle was small, FID increased as refuge distance increased, but (3) if the

escape angle was large, the lizard allowed the predator to approach closer independently of refuge distance.

Table 1. Results of a General Linear Model for the effects of microhabitat, sex and season on refuge distance of *L. schreiberi* lizards.

	ndf, ddf	*F*	*B*	95% CI	*p*	partial η^2
Microhabitat	1, 99	1.13	−25.06	−71.85–21.72	0.29	0.011
Season	1, 99	1.40	24.67	−16.63–65.99	0.24	0.014
Sex	1, 99	0.02	−2.64	−43.83–38.56	0.90	<0.001
Microhabitat × Season	1, 97	0.62	−38.09	−134.24–58.06	0.43	0.006
Microhabitat × Sex	1, 97	0.12	16.70	−77.84–111.24	0.73	0.001
Season × Sex	1, 97	0.38	−28.10	−118.03–61.82	0.54	0.004

Nonsignificant effects are shown as seen after back-substitution to the final model. For interactions, the single effects in the given interaction were also back-substituted. Degrees of freedom (ndf, ddf), F statistics, parameter estimate (*B*) and its 95% confidence interval (CI), significance level (p) and effect size (partial η^2) are shown.

Discussion

Our results were not entirely consistent with our predictions. While our data revealed strong and complex environmental influence on lizard escape behaviour (FID), we did not find any effect of sex or reproductive condition/mating season. Moreover, none of the environmental variables analysed affected refuge distance. This is, however, interesting because the detectability of lizards by the predator and the attack success should be higher on open rocky substrate than in dense vegetation. Thus, we expected that lizards maintain different distances to potential refuges in different microhabitats, while our results suggest that distance to refuge is not strategic in this lizard species. Lizards rather seemed to move and stop freely through their home ranges and simply adjust FID to the current distance to available refuges in each situation.

In contrast, the environment had several effects on FID, which reflects escape decisions in emergency situations. In several lizard species, individuals have longer FID in areas with low cover because they are more conspicuous there (Snell et al. 1988; Martín and López 1995, 2000a; Majláth and Majláthova 2009). Depending on the microhabitat, *L. schreiberi* lizards showed two different escape tactics. In grassy substrates offering

vegetative cover, FID varied with the refuge distance. However, FID was not related to distance to refuge in rocky substrates without cover. In a meta-analysis with 17 lizard species, a positive correlation between refuge distance and FID was found, and this relation-

Table 2 Results of a General Linear Model for the effects of microhabitat, sex and season on flight initiation distance (FID) of *L. schreiberi* lizards.

	ndf, ddf	*F*	*B*	95% CI	*p*	partial η^2
Microhabitat	1, 95	23.98	151.04	89.80–212.28	<0.001*	0.20*
Season	1, 94	1.07	19.18	−17.57–55.93	0.303	0.011
Sex	1, 94	0.33	10.08	−24.53–44.69	0.560	0.004
Refuge distance	1, 95	5.03	0.74	0.44–1.04	0.027*	0.05*
Escape angle	1, 95	0.98	0.35	−0.35–1.06	0.325	0.010
Microhabitat × Season	1, 93	0.62	−32.22	−113.69–49.25	0.430	0.007
Microhabitat × Sex	1, 93	0.80	35.45	−43.32–114.22	0.370	0.009
Microhabitat ×Refuge distance	1, 95	20.28	−0.91	−1.31–−0.51	<0.001*	0.176*
Microhabitat ×Escape angle	1, 94	0.002	−0.03	−1.24–1.18	0.960	<0.001
Season × Sex	1, 92	1.17	−40.88	−115.96–34.20	0.282	0.013
Season × Refuge distance	1, 93	0.79	−0.16	−0.51–0.19	0.376	0.008
Season × Escape angle	1, 93	0.24	−0.23	−1.17–0.71	0.627	0.003
Sex× Refuge distance	1, 93	2.60	0.29	−0.07–0.65	0.110	0.027
Sex × Escape angle	1, 93	0.71	0.39	−0.53–1.30	0.40	0.008
Refuge distance ×Escape angle	1, 95	6.81	−0.006	−0.1–−0.001	0.011*	0.070*

Nonsignificant effects are shown as seen after back-substitution to the final model. For interactions, the single effects in the given interaction were also back-substituted. Degrees of freedom (ndf, ddf), F statistics, parameter estimate (B) and its 95% confidence interval (CI), significance level (p) and effect size (partial η2) are shown.

∗ Significant value.

ship was stronger when the variability of refuge distance was higher (Cooper in press). This suggests variation in the flexibility of escape strategies: there may be either low flexibility with individuals choosing preventively similar refuge distances and, thus, reacting to predators similarly, or high flexibility with variable refuge distances and the subsequent refuge-distance-dependent FID. The habitat-dependent escape strategies in our study might be explained by similar grounds if the availability of potential refuges at different distances was higher in grass than in rocky substrates. Alternatively, differences might result from the above-mentioned detectability and predator attack success differences in different microhabitats. Also, thermal differences between exposed and refuge locations in different microhabitats, and their associated thermal costs, might be important (Martín and López 1999b, 2000b, 2010). In sunny, exposed rocky microhabitats the substrate temperature can

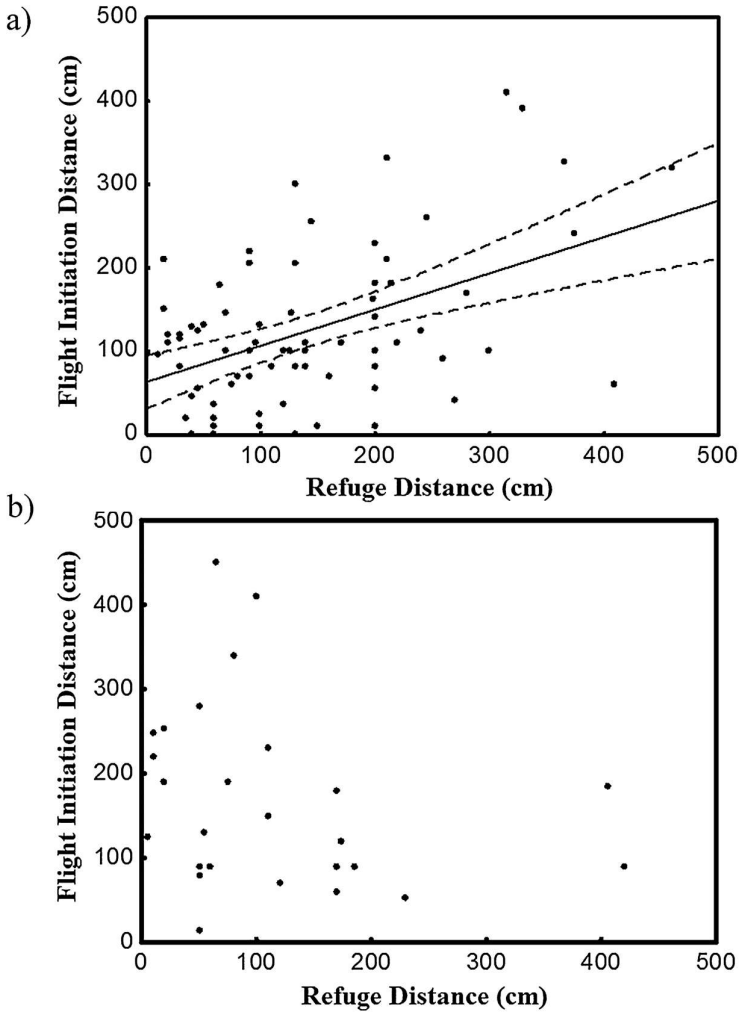

Figure 1. The effect of refuge distance on flight initiation distance (FID) of *L. schreiberi* lizards in (**a**) grassy substrates or (**b**) in open rocky microhabitat types.

be very high, whereas refuge temperature inside rock crevices is low, while in microhabitats covered by vegetation, external and refuge temperatures do not differ as greatly, which could result in more predictable use of refuges and a positive relationship between FID and distance to refuge in vegetated areas.

The relationship between escape angle and refuge distance affected FID in a complex manner. Lizards very close to refuge allowed the predator to approach closer than those farther from refuge, irrespective of escape angle. However, escape angle affected the FID of lizards farther from refuge. If the escape angle was large, the lizard allowed the predator to approach closer, irrespective of the refuge distance. For lizards farther from refuge, FID depended on refuge distance only if the escape angle was small. These results

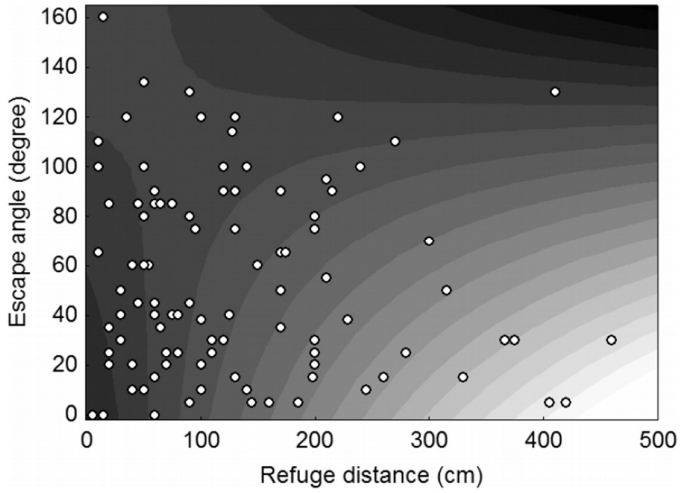

Figure 2. The effects of refuge distance, and escape angle on flight initiation distance (FID) of L. schreiberi lizards (shown in the z-axis with a colour code). The colour codes represent different flight initiation distances (FIDs) ranging from 0 cm (black) to 450 cm (white). The equation of the relationship was: FID = 0.744 × refuge distance + 0.352 × escape angle − 0.006 × refuge distance × escape angle.

were expected because if a lizard is close to refuge, it can delay escape and still reach the refuge safely ahead of the predator even if the lizard has to flee toward the predator to get to refuge. If the escape angle is small (i.e., the lizard flees nearly directly away from the predator), lizards should adjust their FID depending on refuge distance (or time needed to reach it) (Cooper 1997a; Stankovich and Blumstein 2005). However, if the escape angle is large and, thus, fleeing may move the prey closer to the predator, one would intuitively expect prey either flee early when the predator is still far away or rely on crypsis and remain motionless as long as possible. In the skink *Eumeces laticeps* (Cooper 1997a) and the marmot *Marmota monax* (Kramer and Bonenfant 1997) FID increased with escape angle. In the lizard *Holbrookia propinqua*, when the predator approaches on a line that blocked the lizard from fleeing to the closest refuge, the lizard invariably flees toward an alternative refuge (Cooper 1999a). Hence these animals avoided predation risk by escaping early or to alternative refuges, ensuring they can reach their refuge before the predator. Our results were different, hence, it seems that *L. schreiberi* — instead of taking the risk of running towards the predator or to an alternative refuge — let the predator approach closer while relying on crypsis as long as possible. By doing so, they accept a decreased margin of safety (Kramer

and Bonenfant 1996) in exchange for a decreased probability of being detected and attacked. This tradeoff is highly plausible because most individual *L. schreiberi* lizards finally ran directly to the known refuge even if the predator was in that direction, which could still be safer than escaping to an unkown alternative refuge where they may face dangerous encounters with another type of predator (e.g., saurophagous snakes) (Amo et al. 2003, 2005).

Contrary to our expectations, gender or mating season did not affect FID or distance to refuge in *L. schreiberi*. Nevertheless, because our observations in different seasons were not made in the same year, as a result of logistic contraints, there might be a confounding effect of reproductive period with year. Therefore the lack of seasonal difference must be interpreted with caution. With respect to the lack of gender differences, conspicuous animals often suffer higher predation risk (Stuart-Fox et al. 2003; Husak et al. 2006), such as males of the closely related Western green lizard (*Lacerta bilineata*), which are more brightly colored than females and are more often captured by common kestrels, *Falco tinnunculus* (Constantini et al. 2007). Nevertheless, this sex bias could be caused either because males do not compensate for the higher predation risk of having more conspicuous coloration or simply by the higher activity of males in the mating season. The latter was suggested by the absence of a sex difference in predation rate in an experiment using painted epoxy-lizard models of the sexual dichromatic Sand lizards (*L. agilis*), which precluded any differences in activity level (Olsson 1993). However, sexual differences in colour and activity were not associated with a sex difference in FID in *L. schreiberi*.

In a meta-analysis, it was found that there are no differences between males and females in FID in two-thirds of lizard species (Cooper in press), and some of these lizard species have sexual dichromatism, as does our study species. In *Platysaurus broadleyi* FID and refuge distance do not differ between sexes, presumably because there is no difference in predation risk between males and females (Whiting 2002). The lack of sexual FID differences in *L. schreiberi* might be also explained if the more conspicuous males could escape faster than the more cryptic females, as occurs in many other species (Cullum 1998; Lailvaux et al. 2003). However, in the agamid lizard *Phrynocephalus vlangalii*, a species

without sexual dimorphism, FID does not differ between the sexes and is unrelated to individual escape performance capacity, although males flee farther than females (Qi et al. 2014). The reason for lack of gender effect in *L. schreiberi* could be more complex if opposite constraints affect escape decisions. Cryptic coloration of female *L. schreiberi* does not change seasonally, so females can always use the same effective crypsis and an associated short FID. In contrast, during the mating season coloration of males is more conspicuous, which should initially require longer FID, but conflicting reproductive requirements (i.e., mate searching, territorial defense, mate guarding, etc.) may force males to be more active and take more risk by adopting shorter FID (Magnhagen 1991; Cooper 1997b, 1999b; Cooper and Wilson 2007). Finally, after the mating season, there are no reproductive constraints and coloration of male *L. schreiberi* changes to being less conspicuous, which may allow males to be bolder and have shorter FID. Alternatively, only variation in environmental factors might be the determinants of escape behaviour in this species.

In conclusion, we have found that the escape strategy of *L. schreiberi* is fine-tuned to environmental variation, with FID being adjusted to distance to the refuge on grassy substrates, but not in rocky microhabitats, with the relative positions of the predator, the prey and the refuge being also important determining FID. However, contrary to our expectations, gender and season did not modify escape decisions dictated by the environment. Likely, this statement could change if the animals have other constraints, such as nearby presence of food or conspecifics, which can result in lost opportunities after escaping (Cooper, 1997b, 1999b, 2000; Cooper and Wilson, 2007). Future studies are needed to examine whether the conspicuous nuptial coloration of male *L. schreiberi* does not increase predation risk, or whether males just accept the predation costs of their ornaments due to conflicting reproductive requirements.

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References

Amo L, López P, Martín J (2003) Risk level and thermal costs affect the choice of escape strategy and refuge use in the wall lizard, *Podarcis muralis*. Copeia 2003:899-905

Amo L, López P, Martín J (2005) Flexibility in antipredatory behavior allows wall lizards to cope with multiple types of predators. Ann Zool Fenn 42:109-121

Braña F (1993) Shifts in body-temperature and escape behavior of female *Podarcis muralis* during pregnancy. Oikos 66:216-222

Bulova SJ (1994) Ecological correlates of population and individual variation in antipredator behavior of two species of desert lizards. Copeia:980-992

Cabido C, Galán P, López P, Martín J (2009) Conspicuousness-dependent antipredatory behavior may counteract coloration differences in Iberian rock lizards. Behav Ecol 20: 362-370

Carrascal LM, López P, Martín J, Salvador A (1992) Basking and antipredator behaviour in a high altitude lizard: implications of heat-exchange rate. Ethology 92:143-154

Cohen J (1992) A power primer. Psychol Bull 112:155-159

Cooper WE (1997a) Escape by a refuging prey, the broad-headed skink (*Eumeces laticeps*). Can J Zool 75:943-947

Cooper WE (1997b) Factors affecting risk and cost of escape by the broad-headed skink (*Eumeces laticeps*): predator speed, directness of approach, and female presence. Herpetologica 53:464-474

Cooper WE (1997c) Threat factors affecting antipredatory behavior in the broad-headed skink (*Eumeces laticeps*): repeated approach, change in predator path, and predator's field of view. Copeia 1997:613-619

Cooper WE (1998a) Effects of refuge and conspicuousness on escape behavior by the broad-headed skink (*Eumeces laticeps*). Amphib-Reptil 19:103-108

Cooper WE (1998b) Direction of predator turning, a neglected cue to predation risk. Behaviour 135:55-64

Cooper WE (1999a) Escape behavior by prey blocked from entering the nearest refuge. Can J Zool 77:671-674

Cooper WE (1999b) Tradeoffs between courtship, fighting, and antipredatory behavior by a lizard, *Eumeces laticeps*. Behav Ecol Sociobiol 47:54-59

Cooper WE (2000) Tradeoffs between predation risk and feeding in a lizard, the broad-headed skink (*Eumeces laticeps*). Behaviour 137:1175-1189

Cooper WE (in press) Reptiles. In: Cooper WE, Blumstein DT (eds) Escaping from predators: an integrative view of escape decisions and refuge use. Cambridge University Press, Cambridge

Cooper WE, Frederick WG (2007) Optimal flight initiation distance. J Theor Biol 244:59-67

Cooper WE, Wilson DS (2007) Sex and social costs of escaping in the striped plateau lizard *Sceloporus virgatus*. Behav Ecol 18:764-768

Costantini D, Brune E, Fanfani A, Dell'Omo G (2007) Male-biased predation of western green lizards by Eurasian kestrels. Naturwissenschaften 94:1015-1020

Cuadrado M, Martín J, López P (2001) Camouflage and escape decisions in the common chameleon *Chamaeleo chamaeleon*. Biol J Linn Soc 72:547-554

Cullum AJ (1998) Sexual dimorphism in physiological performance of whiptail lizards (genus *Cnemidophorus*). Phys Zool 71:541-552

Dill LM, Houtman R (1989) The influence of distance to refuge on flight initiation distance in the grey squirrel (*Sciurus carolinensis*). Can J Zool 67:233-238

Domenici P, Blagburn JM, Bacon JP (2011a) Animal escapology I: theoretical issues and emerging trends in escape trajectories. J Exp Biol 214:2463-2473

Domenici P, Blagburn JM, Bacon JP (2011b) Animal escapology II: escape trajectory case studies. J Exp Biol 214:2474-2494

Endler JA (1991) Interactions between predators and prey. In: Krebs JR, Davies NB (eds) Behavioural ecology: an evolutionary approach, 3rd edn. Blackwell, Oxford, pp 169–196

Forsman A, Appelqvist S (1998) Visual predators impose correlated selection on prey color pattern and behavior. Behav Ecol 9:409-413

Godin JGJ, Dugatkin LA (1996) Female mating preference for bold males in the guppy, *Poecilia reticulata*. P Natl Acad Sci USA 93:10262-10267

Heatwole H (1968) Relationship of escape behaviour and camouflage in anoline lizards. Copeia 1968:109-113

Husak JF, Macedonia JM, Fox SF, Sauceda RC (2006) Predation cost of conspicuous male coloration in collared lizards (*Crotaphytus collaris*): an experimental test using clay-covered model lizards. Ethology 112:572-580

Johnson CR (1970) Escape behavior and camouflage in two subspecies of *Sceloporus occidentalis*. Am Midl Nat 84:280-282

Kramer DL, Bonenfant M (1997) Direction of predator approach and the decision to flee to a refuge. Anim Behav 54:289-295

Lailvaux SP, Alexander GJ, Whiting MJ (2003) Sex-based differences and similarities in locomotor performance, thermal preferences, and escape behaviour in the lizard *Platysaurus intermedius wilhelmi*. Phys Biochem Zool 76:511-521

Lima SL (1998) Stress and decision making under the risk of predation: recent developments from behavioral, reproductive, and ecological perspectives. Adv Stud Behav 27: 215-290

Lima SL, Dill LM (1990) Behavioral decisions made under the risk of predation: a review and prospectus. Can J Zool 68:619-640

Lindström L, Ahtiainen JJ, Mappes J, Kotiaho JS, Lyytinen A, Alatalo RV (2007) Negatively condition dependent predation cost of a positively condition dependent sexual signalling. J Evol Biol 19:649-656

Magnhagen C (1991) Predation risk as a cost of reproduction. Trends Ecol Evol 6: 183-186

Majláth I, Majláthová V (2009) Escape behavior of the green lizard (*Lacerta viridis*) in the Slovak Karst. Acta Ethol 12:99-103

Marco A, Pérez-Mellado V (1999) Mate-guarding, intrasexual competition and the mating success in males of the non-territorial lizard *Lacerta schreiberi*. Ethol Ecol Evol 11:279-286

Martín J, López P (1995) Influence of habitat structure on escape tactics of the lizard *Psammodromus algirus*. Can J Zool 73:129-132

Martín J, López P (1996) The escape response of juvenile *Psammodromus algirus* lizards. J Comp Psychol 110:187-192

Martín J, López P (1999a) Nuptial coloration and mate guarding affect escape decisions of male lizards, *Psammodromus algirus*. Ethology 105:439-447

Martín J, López P (1999b) When to come out from a refuge: risk-sensitive and state-dependent decisions in an alpine lizard. Behav Ecol 10:487-492

Martín J, López P (2000a) Fleeing to unsafe refuges: effects of conspicuousness and refuge safety on the escape decisions of the lizard *Psammodromus algirus*. Can J Zool 78:265-270

Martín J, López P (2000b) Costs of refuge use affect escape decisions of Iberian rock lizards, *Lacerta monticola*. Ethology 106:483-492

Martín J, López P (2003) Changes in the escape responses of the lizard *Acanthodactylus erythrurus* under persistent predatory attacks. Copeia 2003:408-413

Martín J, López P (2009) Multiple color signals may reveal multiple messages in male Schreiber's green lizards, *Lacerta schreiberi*. Behav Ecol Sociobiol 63:1743-1755

Martín J, López P (2010) Thermal constraints of refuge use by Schreiber's green lizards, *Lacerta schreiberi*. Behaviour 147:275-284

Martín J, López P, Cooper WE (2003) Loss of mating opportunities influences refuge use in the Iberian rock lizard, *Lacerta monticola*. Behav Ecol Sociobiol 54:505-510

Møller AP, Christiansen SS, Mousseau TA (2011) Sexual signals, risk of predation and escape behavior. Behav Ecol 22:800-807

Murtaugh PA (2009) Performance of several variable-selection methods applied to real ecological data. Ecol Lett 12:1061-1068

Olsson M (1993) Nuptial coloration and predation risk in model sand lizards, *Lacerta agilis*. Anim Behav 46:410-412

Qi Y, Noble DWA, Wu Y, Whiting MJ (2014) Sex- and performance-based escape behaviour in an Asian agamid lizard, *Phrynocephalus vlangalii*. Behav Ecol Sociobiol 68:2035-2042

Salvador A (1988) Selección de microhábitat del lagarto verdinegro (*Lacerta schreiberi*) (Sauria, Lacertidae). Amphib-Reptil 9:265-276

Salvador A (2014) *Lacerta schreiberi* Bedriaga, 1878. In: Salvador A (ed) Reptiles, fauna Ibérica, Vol. 10, 2nd edn. Museo Nacional de Ciencias Naturales, CSIC, Madrid, pp 488-501

Smith DG (1997) Ecological factors influencing the antipredator behaviors of the ground skink, *Scincella lateralis*. Behav Ecol 8:622-629

Smith GR (1996) Correlates of approach distance in the striped plateau lizard (*Sceloporus virgatus*). Herpetol J 6:56-58

Snell HL, Jennings RD, Snell HM, Harcourt S (1988) Intrapopulation variation in predator-avoidance performance of Galapagos lava lizards: the interaction of sexual and natural selection. Evol Ecol 2:353-369

Stankowich T, Blumstein DT (2005) Fear in animals: a review and meta-analysis of risk assessment. Proc R Soc Lond B 272:2627-2634

Stuart-Fox DM, Moussalli A, Marshall NJ, Owens IPF (2003) Conspicuous males suffer higher predation risk: visual modelling and experimental evidence from lizards. Anim Behav 66:541-550

Whiting MJ (2002) Field experiments on intersexual differences in predation risk and escape behaviour in the lizard *Platysaurus broadleyi*. Amphib-Reptil 23:119-124

Ydenberg RC, Dill LM (1986) The economics of fleeing from predators. Adv Stud Behav 16:229-249

Zani PA, Jones TD, Neuhaus RA, Milgrom JE (2009) Effect of refuge distance on escape behavior of side-blotched lizards (*Uta stansburiana*). Can J Zool 87:407-414

Chapter II

This chapter reproduces entirely the manuscript:

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Relative contribution of dietary carotenoids and vitamin E to visual and chemical sexual signals of male Iberian green lizards: an experimental test

Renata Kopena, Pilar López and José Martín

Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain

Abstract

Carotenoid-based sexual ornaments are widespread, but the role of carotenoids as honest signalers in the trade-off between coloration and antioxidant protection remains controversial. It has been suggested that the function of carotenoids might not be an antioxidant per se, but that colorful carotenoids may indirectly reflect the levels of nonpigmentary antioxidants, such as melatonin or vitamin E. We experimentally fed male Iberian green lizards (*Lacerta schreiberi*) additional carotenoids or vitamin E alone, or a combination of carotenoids and vitamin E dissolved in soybean oil, whereas a control group only received soybean oil. We examined the effects of the dietary supplementations on characteristics of lizard sexual coloration and of the chemical profile of femoral gland secretions. Results indicated that both carotenoids and vitamin E in the diet increased the expression of some visual signals (head and chest, but not dorsum) in comparison to controls. However, different traits were differentially affected, and in many cases, the addition of vitamin E, alone or in combination with carotenoids, had a greater effect on the expression of coloration than the addition of carotenoids alone, even for carotenoid-dependent

ornaments. Our results support the idea that other nonpigmentary antioxidants, such as vitamin E, are needed in addition to carotenoids to increase the expression of coloration of *L. schreiberi* lizards. Therefore, coloration may only indirectly reflect the levels of nonpigmentary antioxidants. In contrast, an increase in dietary nonpigmentary antioxidant vitamin E was directly reflected in the chemical signals. Because of an observed concordance between visual and chemical signals, we suggest that both may be used in different contexts albeit conveying similar messages in different sensory channels.

Keywords: Sexual coloration · Femoral secretions · Antioxidants · Lizards · Sexual ornaments

Introduction

Colorful sexual ornaments are widespread among animals, and in many cases, these traits are used as honest signals of health and condition in sexual selection (Andersson 1994; Olson and Owens 1998). In fact, many studies have suggested that only healthier males may afford to produce more elaborate visual colorful displays (e.g., Blount et al. 2003; Faivre et al. 2003). The honesty of such signals is based on dietary carotenoids which are costly to obtain resulting in a trade-off between allocation of carotenoids to the sexual signal and/or to antioxidant defense (von Schantz et al. 1999; Møller et al. 2000; McGraw and Ardia 2003; Alonso-Alvarez et al. 2004; Svensson and Wong 2011).

However, the role of carotenoids as honest signalers in the trade-off between coloration and antioxidant protection remains controversial (e.g., Costantini et al. 2007; Isaksson et al. 2007; Pérez-Rodríguez 2009; Hill and Johnson 2012). A recent meta-analysis in birds provides support for the relationships of trait coloration and circulating carotenoid level with different measures of immunocompetence and oxidative stress, but the effect sizes are low, suggesting that these relationships might not be causal and that complementary mechanisms maintaining honesty might be involved (Simons et al. 2012). In this context,

Hartley and Kennedy (2004) suggested the alternative hypothesis that the biological role of carotenoids might not be as antioxidants per se, but that colorful carotenoids would just reflect and provide "information" on the true organismal antioxidants, such as the level of melatonin, vitamin C, or vitamin E (Burton and Traber 1990; Brigelius-Flohe and Traber 1999; Martínez et al. 2008). This may be explained because oxidation of carotenoids alters or destroys their color (Woodall et al. 1997). Therefore, a male only may show carotenoids with their color intensity preserved if he also has other types of efficient antioxidants needed for the protection of carotenoids (Hartley and Kennedy 2004).

Some studies have tested the main prediction of this hypothesis, i.e., that increasing the availability of nonpigmentary antioxidants should enhance the expression of carotenoid-based sexual traits. For example, carotenoid and melatonin dietary supplementation have an additive effect on bill color of male zebra finches (Bertrand et al. 2006). Male sticklebacks that received a diet with high levels of the colorless antioxidant vitamins C and E produced more intensely colored (but not larger) carotenoid-based nuptial coloration (Pike et al. 2007). Male gulls that received vitamin E supplements had larger red spots in the bill than control birds, although color intensity did not change (Pérez et al. 2008). These studies suggest that carotenoid-based ornaments may honestly signal an individual's availability of non-carotenoid antioxidants. However, in male greenfinches, vitamin E supplementation did not affect carotenoid-based plumage coloration (Karu et al. 2008).

Many lizards have evolved colorful sexually dimorphic ornaments that are used in intraspecific behaviors (reviewed in Cooper and Greenberg 1992). The size and color characteristics of male visual badges may determine success in male– male competition (Thompson and Moore 1991; Olsson 1994; Martín and Forsman 1999; Whiting et al. 2006; Bajer et al. 2011) or mate acquisition (Kwiatkowski and Sullivan 2002; Anderholm et al. 2004; Hamilton and Sullivan 2005; Salvador et al. 2008; Bajer et al. 2010). In addition, chemical signals (pheromones) also play an important role in intraspecific communication of many lizards (reviewed in Mason 1992; Mason and Parker 2010; Martín and López 2011). Chemical signals may provide detailed information on morphological traits and health

condition of the signaler (López et al. 2006; Martín et al. 2007) and is consequently often used in male–male interactions (López and Martín 2002, 2011; Carazo et al. 2007; Martín and López 2007) as well as in female mate choice (Martín and López 2000, 2006a, b; Olsson et al. 2003; Martín et al. 2007; Kopena et al. 2011). Therefore, many species of lizards appear to base their sexual selection processes on multiple signals (visual and chemical) perceived in different sensorial channels (Martín and López 2010). Moreover, in at least some lizards, the information provided by visual and chemical signals is congruent, suggesting that there may be similar trade-offs maintaining honesty of both chemical and visual signaling (Martín and López 2010).

In some lizards, carotenoid supplementation increased carotenoid plasma concentration (Fitze et al. 2009), but manipulation of carotenoid availability does not always affect the expression of carotenoid-based ornaments (Olsson et al. 2008; Fitze et al. 2009; Steffen et al. 2010; San José et al. 2012). However, carotenoid-based colors have been shown to reflect stress responses in common lizards (Fitze et al. 2009) and health state in common wall lizards (Martín et al. 2008). Moreover, challenges of the immune system result in duller ventrolateral coloration and a decrease of yellow pigments in Iberian wall lizards (López et al. 2009). However, carotenoid intake does not reduce circulating levels of reactive oxygen species or baseline superoxide, suggesting that carotenoids are inefficient antioxidants in vivo and, therefore, are unlikely to provide a direct link between oxidative stress and coloration (Olsson et al. 2008). A recent study shows that integumentary components (i.e., iridophores) other than carotenoids account for condition-dependent chromatic variation of carotenoid-based ornaments in the lizard *Lacerta vivipara* and that changes in iridophore reflectance are probably regulated by vitamin A_1 (San José et al. 2013). Thus, additional physiological mechanisms, such as the role of nonpigmentary antioxidant vitamins A or E, may be involved in lizard coloration.

The Iberian or Schreiber's green lizard (*Lacerta schreiberi*) is a large sexually dimorphic lacertid from the Iberian Peninsula. Males have, especially during the breeding season, green dorsal coloration with small black spots, yellow chest and ventral coloration,

and bright blue and UV throat and mental coloration (Martín and López 2009). In contrast, females are mainly dull brown, with pale yellow vents. Interindividual variation in the characteristics of coloration of males can be related to variation in morphology, health state, dominance, and pairing status, but different relationships, probably based on different physiological trade-offs, are found for each color signal (Martín and López 2009). Furthermore, during the mating season, male *L. schreiberi* have well developed femoral hindlimb glands that produce abundant secretions containing numerous lipophilic compounds, including several steroids, fatty acids, alcohols, and, interestingly, large amounts of α-tocopherol (=vitamin E) (López and Martín 2006). In the closely related European green lizard (*L. viridis*), males produce similar secretion compounds (Kopena et al. 2009), and females preferred to use areas scent-marked by males with experimentally increased vitamin E secretion levels (Kopena et al. 2011). This suggests that the cost of allocating antioxidant vitamin E to secretions may confer reliability to chemical signals of green lizards.

In this study, we experimentally fed male lizards L. schreiberi with additional carotenoids or vitamin E alone, or a combination of carotenoids and vitamin E. Compared with a control group, we examined the effects of this dietary supplementation on characteristics of throat, chest, and dorsal coloration, and on the chemical profile of femoral gland secretions of lizards. If the antioxidant properties of vitamin E protected carotenoids from oxidation, we predicted that lizards supplemented with vitamin E should have brighter colorations than lizards not supplemented with vitamin E and that lizards supplemented with the combination of carotenoids and vitamin E should have brighter colorations than lizards supplemented with carotenoids alone. With respect to femoral secretions, we predicted that lizards supplemented with vitamin E should allocate higher proportions of vitamin E to their femoral secretions, whereas lizards supplemented with carotenoids alone should not differ from the control lizards.

Materials and methods

Study animals

In April 2011, we collected 52 adult male lizards *L. schreiberi* at 'Valle de La Fuenfría' and 'Valle de Navalmedio' in the Guadarrama mountains (40°44′ N, 4°02′ W; Madrid Province, Spain). We immediately transferred lizards to "El Ventorrillo" field station of the Museo Nacional de Ciencias Naturales (Madrid province, Spain), 5 km from the capture sites. During all the experiments, lizards were individually kept in outdoor 51×36×28 cm PVC terraria containing coconut fiber substratum and rocks for cover. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*), house crickets (*Acheta domesticus*), and common black crickets (*Gryllus assimilis*) dusted with calcium powder, and water was provided ad libitum. The photoperiod and ambient temperature were those of the surrounding region. We measured male body weight before the experiments with a digital balance to the nearest 0.1 g (mean±SE=26.5±4.9 g; range=18.0–40.5 g) and used a digital caliper to measure snout-to-vent length (SVL) to the nearest 1 mm (mean±SE=98±6 mm; range=85–111 mm).

Experimental supplementation of the diet

To control for differences in body size, we classified males in groups of four based on similar body length (within±2 mm of SVL). In every quartet, we selected randomly which individuals were assigned to each of four treatments: 'carotenoids,' 'vitamin E,' 'combination of carotenoids and vitamin E,' or 'control'. Each male was subjected to daily supplementation for 28 days in his own terraria.

In the carotenoid treatment, males (n=13) were fed daily 8 μg of a mix of β-carotene, α-carotene, zeaxanthin, lutein, and lycopene (in a rate of 5:1.7:0.3:5:5) (Solgar Natural Lutein Lycopene Carotene Complex, purchased from Solgar Inc., Leonia, New Jersey, USA)

dissolved in 20 µL soybean oil. Thus, at the end of the experiment, each male had been provided with a total of 224 µg carotenoids. The daily amount of carotenoids was based on previous studies of carotenoid supplementation in other lizards (Olsson et al. 2008) and corrected for differences in body size between species.

In the vitamin E treatment, males (n=13) were fed daily 20 µL vitamin E supplement (synthetic (±)-α-tocopherol; purchased from Sigma-Aldrich Chemicals Co., St. Louis, Missouri, USA), which contained 97 % of synthetic vitamin E (approx. 1,014 IU mL^{-1}) and 3 % soybean oil (with approx. 0.32 IU mL^{-1} of natural vitamin E, i.e., D-α-tocopherol). Thus, the daily dose provided for each male was approximately 20.2 IU of vitamin E, which is close to the daily minimal physiological necessity of vitamin E for similar-sized reptiles, and well below the tolerable upper intake levels (Mader 1996; Allen et al. 2004; Bender 2009).

In the third treatment using a combination of carotenoids and vitamin E, male lizards (n=13) were fed daily 20 µL of vitamin E and 8 µg of carotenoids as above, both dissolved in 20 µL soybean oil. Finally, in the control treatment, males (n=13) were fed daily 20 µl soybean oil. To ensure that all lizards ingested the same amount of vitamin or carotenoid supplement or the control solution, we gently handled lizards and used sterile plastic syringes with a canula to slowly deliver the solution into their mouth, thus ensuring that lizards swallowed the entire dose.

Color measurements

At the end of the dietary supplementation experiment, we measured reflectance of lizards' coloration from 300 to 700 nm using an Ocean Optics USB2000 spectroradiometer with a DT-1000-MINI Deuterium–Halogen light source (Ocean Optics, Inc., Dunedin, FL, USA). This range of wavelengths coincides with the spectral sensitivity measured for other lizard species, which includes visual perception in the UV range (e.g., Fleishman et al. 1993; Loew et al. 2002). To exclude ambient light and standardize measuring distance, a cylindrical metallic tube was mounted on the bifurcated fiber optic probe (Montgomerie

2006). The probe was held at a 90° angle to the skin, and reflectance was always measured by the same person (PL). We measured coloration of three visually distinct large patches of coloration ("blue" throat, "yellow" chest, and "green" dorsum) at three standardized spots: the middle of the throat (between the last chin shields and the collar; "throat"), the chest (just anterior to the two forelimbs at the middle of the second row of scales prior to the collar; "chest"), and the dorsum (in the middle point above the two forelimbs; "dorsal").

We mathematically summarized the spectra using principal component analyses (PCAs) (Endler 1990; Cuthill et al. 1999; Grill and Rush 2000). The PCA summarizes all of the information about the shape of complex reflectance spectra, including bimodal ones like those found in *L. schreiberi* (see Martín and López 2009) into a few independent PCs (Montgomerie 2006). In PCA of spectral data, PC1 represents here variation in intensity of coloration or brightness, and subsequent PCs represent combinations of hue and chroma (Endler 1990; Cuthill et al. 1999; Grill and Rush 2000). Moreover, the PCA identifies those sections of the spectrum (wavelength regions) that are contributing to the observed variation, independently of their impact in terms of contribution to the total amount of reflectance (Montgomerie 2006). Principal component analyses were performed separately for each part of the body (throat, chest, and dorsal) including spectra for all males. We used one-way analyses of variance (ANOVAs) to compare the characteristics of coloration of each trait defined by the PC scores between treatments. Post hoc comparisons used Tukey's tests (Sokal and Rohlf 1995).

In addition, to clarify the role of carotenoids in coloration, from the raw spectral reflectance data of each body part, we calculated the carotenoid chroma $[(R_{700nm}-R_{450nm})/R_{700nm}]$, which represents relative reflectance around peak absorbance of carotenoids, thus indicating levels of carotenoid pigments incorporated into the integument (Johnsen et al. 2003; Peters et al. 2004). We subsequently correlated carotenoid chroma with the PCscores to explore whether variation in coloration defined by these PCscores could be determined by carotenoid levels.

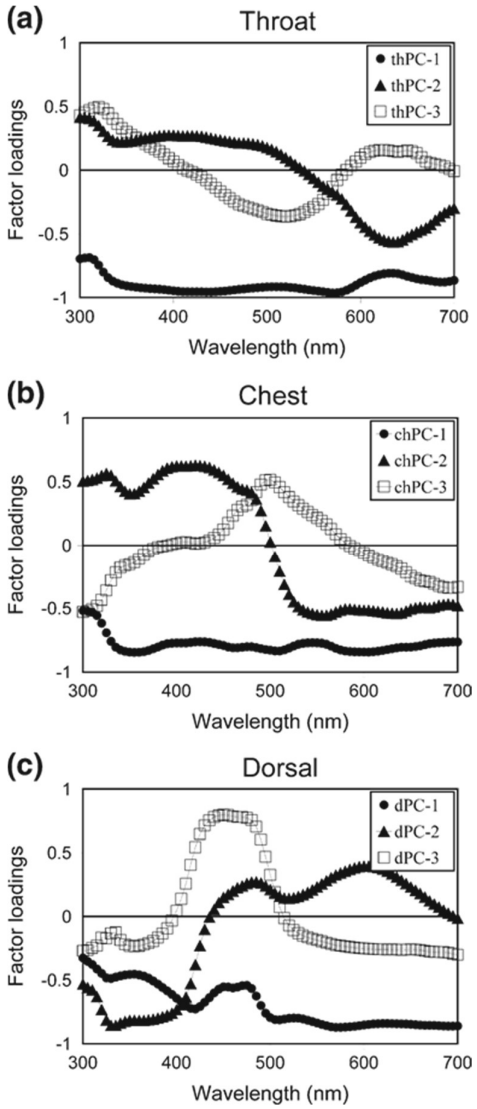

Fig. 1 Coefficients of the first three principal components from PCAs on reflectance spectra that characterize throat (**a**), chest (**b**), and dorsal (**c**) coloration of male lizards *L. schreiberi.*

Chemical analyses of femoral secretions

At the end of the experiments, we collected femoral secretion of males directly into glass vials with glass inserts, later closed with Teflon-lined stoppers and stored at −20 °C. Samples were analyzed by gas chromatography-mass spectrometry (Finnigan-ThermoQuest GC-MS Trace 2000, Thermo-Quest Corporation, Austin, Texas) equipped with a Supelco-Equity-5 (Bellefonte, PA) column temperature programmed (50–280°C at 5°C/min and 280°C for 30 min). Compounds were identified by comparison of mass spectra in the NIST/EPA/NIH (NIST 02, www. nist.gov) library and later confirmed with authentic

standards (see López and Martín 2006 for details of analyses and chemical compounds in secretions of this lizard species). The relative amount of each compound was determined as the percent of the total ion current area transformed following Aitchison's formula: [Zij=ln(Yij/$g(Yj)$)], where Zij is the standardized peak area i for individual j, Yij is the peak area i for individual j, and $g(Yj)$ is the geometric mean of all peaks for individual j (Aitchison 1986) (for similar analyses, see López et al. 2006).

We subsequently calculated Euclidean distances between every pair of individual samples to produce a resemblance matrix that formed the basis of the analyses. Differences between treatments were investigated using canonical analysis of principal coordinates (CAP, Anderson and Willis 2003). We further used a single-factor permutational multivariate analysis of variance test (PERMANOVA, Anderson 2001; McArdle and Anderson 2001) based on the Euclidean resemblance matrix using 9,999 permutations to analyze whether the composition of the femoral secretions varied between treatments after the diet supplementation. Pairwise comparisons were made with permutation tests. The software PRIMER V6.1.13 (Clarke and Gorley 2006) with the PERMANOVA+V1.0.3 add-on package (Anderson et al. 2008) was used to investigate differences between chemical profiles.

Results

Throat coloration

The PCA on reflectance data of all spectra of throat coloration (Fig. 1a) produced three throat principal components (thPCs) that together accounted for 96.7 % of the variation in the original spectra. The first PC (thPC1) accounted for 80.6 % of variation (eigenvalue=65.33). Coefficients relating thPC1 to the original reflectance data were all negative and of similar magnitude (Fig. 1a), so thPC1 represented achromatic brightness variation in the original spectra. The second PC (thPC2) accounted for a further 10.1 % of

the variation (eigenvalue=8.13) in the original spectra. The coefficients relating thPC2 to the original reflectance values below 540 nm were all positive while, above 540 nm, they were negative (Fig. 1a). The thPC2 was highly and negatively correlated with throat carotenoid chroma ($r=-0.67$, $F_{1,47}=38.65$, $P<0.0001$). Thus, thPC2 represented variation in the relative amount of short- to long-wavelength reflectance, with greater thPC2 scores indicating more saturated "bluish" colors with less carotenoids. The third PC (thPC3) accounted for 6.0 % of the variation (eigenvalue=4.90), and the pattern of coefficients suggested it represented variation in the relative amounts of medium (410–580 nm) wavelengths in the negative side to both very short (300–410 nm) and very long (580–700 nm) wavelengths in the positive side (Fig. 1a). There were significant differences between treatments in thPC1 (one-way ANOVA; $F_{3,45}=4.47$, $P=0.008$) (Figs. 2a and 3a), indicating that lizards supplemented with a combination of carotenoids and vitamin E had significantly brighter throat coloration than control lizards (Tukey's tests, $P=0.004$). The throat coloration of males supplemented with carotenoids alone or vitamin E alone did not differ ($P=0.93$) but had significantly brighter throat coloration than control lizards ($P<0.05$, respectively). In contrast, there were no significant differences between treatments in characteristics of throat coloration described by thPC2 (i.e., proportion of short- to long-wavelengths) (one-way ANOVA; $F_{3,45}=0.62$, $P=0.61$) (Figs. 2a and 3b), or by thPC3, i.e., proportions of medium to both very short- and very long-wavelengths (one-way ANOVA; $F_{3,45}=1.94$, $P=0.14$) (Figs. 2a and 3c).

Chest coloration

The PCA on reflectance data of all spectra of chest coloration (Fig. 1b) produced three chest principal components (chPCs) that together accounted for 94.7 % of the variation in the original spectra. The first PC (chPC1) accounted for 62.3 % of variation (eigenvalue=50.47). The coefficients relating chPC1 to the original reflectance data were all negative and of similar magnitude (Fig. 1b), so chPC1 represented achromatic brightness variation in the original spectra. The second PC (chPC2) accounted for a further 25.5 % of the variation (eigenvalue=20.62) in the original spectra. The coefficients relating chPC2 to

the original reflectance values below 500 nm were all positive while, above 500 nm, they were negative (Fig. 1b). The chPC2 was highly and negatively correlated with chest carotenoid chroma ($r=-0.87$, $F_{1,47}=144.26$, $P<0.0001$). Thus, chPC2 represented variation in the relative amount of short- to long-wavelength reflectance, with lower chPC2 scores indicating more saturated "yellowish" colors probably due to carotenoids. The third PC (chPC3) accounted for 6.9 % of the variation (eigenvalue= 5.63), and the pattern of coefficients suggested it represented variation in the relative amounts of medium (390–580 nm) wavelengths in the positive side to both very short (300– 390 nm) and very long (580–700 nm) wavelengths in the negative side (Fig. 1b).

There were no significant differences between treatments in the characteristics of chest coloration described by the chPC1 (i.e., brightness) (one-way ANOVA; $F_{3,45}=1.10$, $P=0.36$) (Figs. 2b and 4a). However, there were significant differences in chPC2 (one-way ANOVA; $F_{3,45}=5.16$, $P=0.004$) (Figs. 2b and 4b), indicating that lizards supplemented with vitamin E alone had significantly more saturated "yellowish" chest carotenoid-dependent coloration (i.e., greater proportions of long, 500–700 nm wavelengths) than lizards supplemented with carotenoids alone or with a combination of carotenoids and vitamin E (Tukey's tests, $P<0.05$ for both); no significant difference were observed among the two latter treatments ($P=0.99$). However, the chest coloration of males supplemented with carotenoids, vitamin E, and a combination of carotenoids and vitamin differed significantly from that of controls ($P<0.05$ in all comparisons; Figs. 2b and 4b).

We also observed significant differences between treatments in chPC3 (one-way ANOVA; $F_{3,45}=2.91$, $P=0.044$). Males supplemented with vitamin E alone or with a combination of carotenoids and vitamin E did not differ (Tukey's test, $P=0.98$), but both groups had significantly greater proportions ($P=0.03$ for both) of medium wavelengths (390–580 nm) in chest coloration compared with lizards supplemented with carotenoids alone and controls. No significant difference were observed among the two latter groups ($P= 0.99$) (Figs. 2b and 4c).

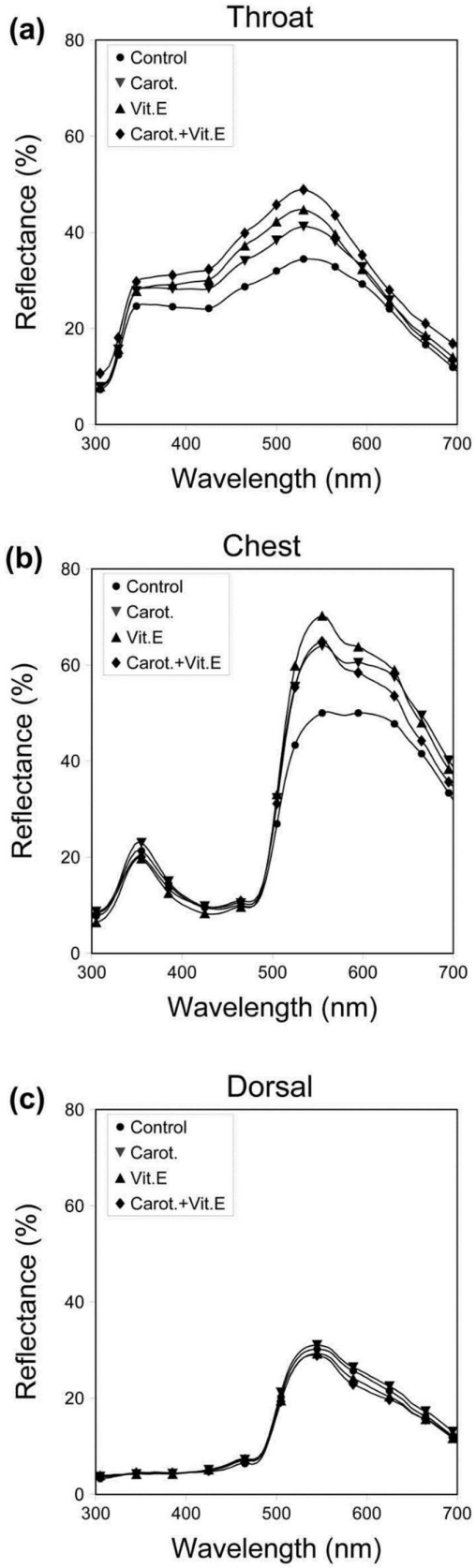

Fig. 2 Mean reflectance of throat (**a**), chest (**b**), and back (**c**) coloration of male lizards *L. schreiberi* at the end of each experimental treatment.

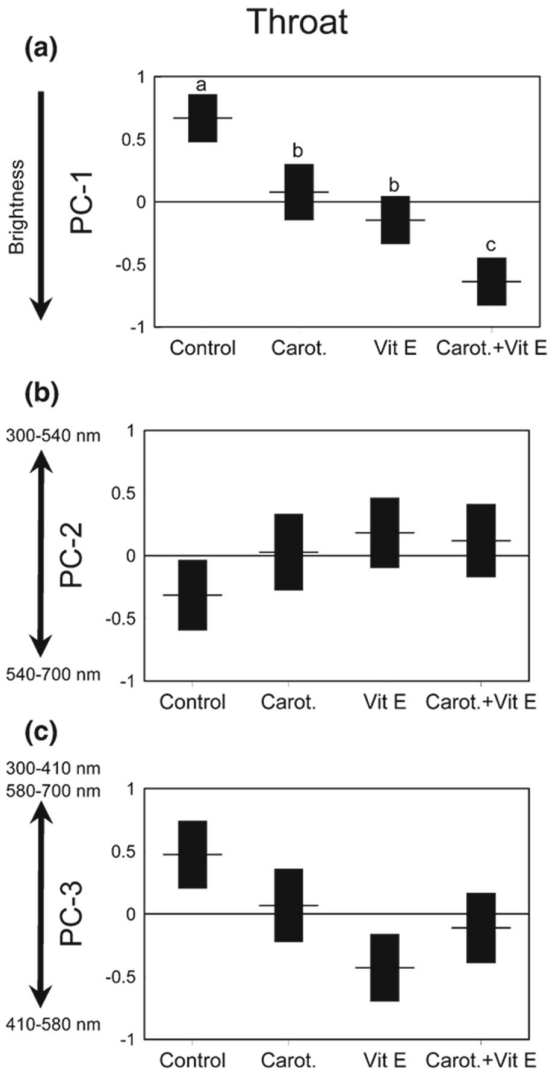

Fig. 3 Mean(±SE) of thPC scores describing characteristics of throat coloration of male lizards *L. schreiberi* at the end of each experimental treatment. Arrows indicate the variables or wavelengths correlated with each PC. Means with the same letter above the bars were not significant different.

Dorsal coloration

The PCA on reflectance data of all spectra of dorsal coloration (Fig. 1c) produced three dorsal principal components (dPCs) that together accounted for 87.1 % of the variation in the original spectra. The first PC (dPC1) accounted for 51.7 % of variation (eigenvalue=41.88). The coefficients relating dPC1 to the original reflectance data were all negative and of similar magnitude (Fig. 1c), so dPC1 represented achromatic brightness variation in the original spectra. The second PC (dPC2) accounted for a further 21.0 % of the variation (eigenvalue= 17.03) in the original spectra. The pattern of coefficients

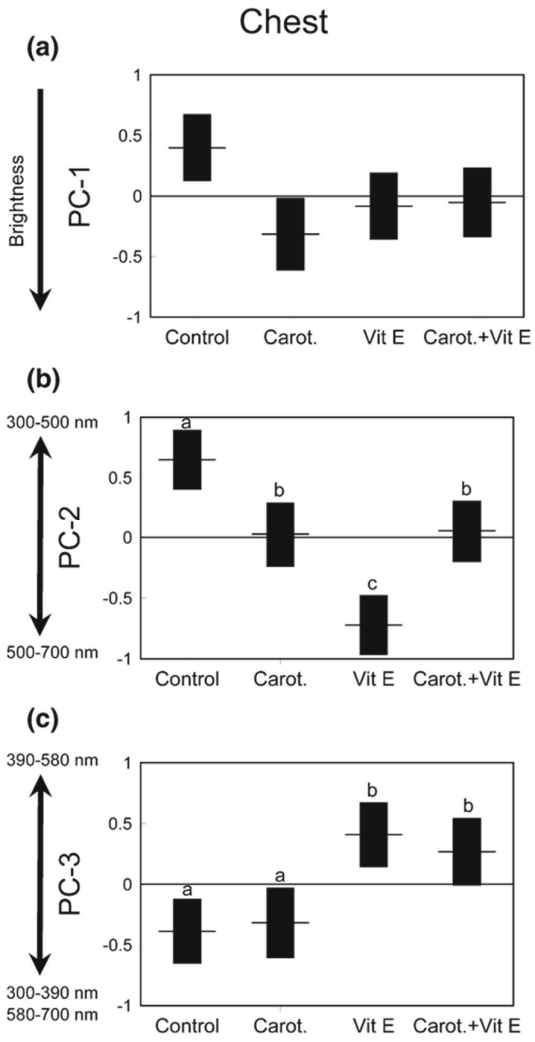

Fig. 4 Mean(±SE) of chPC scores describing characteristics of chest coloration of male lizards *L. schreiberi* at the end of each experimental treatment. Arrows indicate the variables or wavelengths correlated with each PC. Means with the same letter above the bars were not significant different.

suggested it represented variation in the relative amounts of long (440–700 nm) wavelengths in the positive side to short (300–440 nm) wavelengths in the negative side (Fig. 1c). The dPC2 was not significantly correlated with dorsal carotenoid chroma ($r=-0.11$, $F_{1,47}=0.56$, $P=0.46$). Thus, dPC2 represented variation in the relative amount of short- to long-wavelength reflectance, with lower dPC2 scores indicating more saturated "greenish" coloration, which probably was not mainly dependent on carotenoids. The third PC (dPC3) accounted for 14.4% of the variation (eigenvalue=11.68), and the pattern of coefficients suggested it represented variation in the relative amounts of medium (400–510 nm) wavelengths in the positive side to both very short (300–410 nm) and very long (510–700

nm) wavelengths in the negative side (Fig. 1c), with greater dPC3 scores indicating more saturated "greenish" colors.

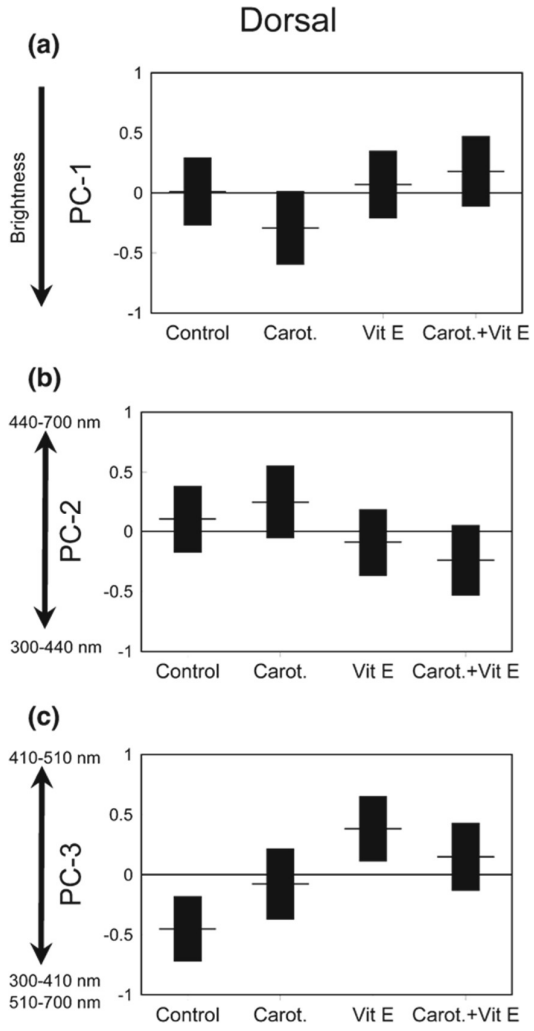

Fig. 5 Mean(±SE) of dPC scores describing characteristics of dorsal coloration of male lizards *L. schreiberi* at the end of each experimental treatment. Arrows indicate the variables or wavelengths correlated with each PC.

There were no significant differences between treatments in any of the characteristics of dorsal coloration described by the PC scores (one-way ANOVAs; dPC1, $F_{3,45}$=0.45, P=0.72; dPC2, $F_{3,45}$ = 0.53, P= 0.67; dPC3, $F_{3,45}$ = 1.70, P= 0.18) (Figs. 2c and 5). Therefore, the supplementation of vitamin E or carotenoids, alone or combined, did not affect male dorsal coloration.

Femoral secretions

The CAP analysis classified 67.3 % of the chemical profiles into the correct treatment using leave-one-out cross-validation and m=33 axes (δ_1^2 =0.96, P=0.001; Fig. 6). The degree of successful classification was higher for the vitamin E alone treatment (76.9 %) than for the carotenoids plus vitamin E (69.2 %), control (69.2 %), or carotenoids alone treatments (53.8 %). The PERMANOVA based on the resemblance matrix comparing the chemical profiles of male femoral secretions between treatments showed statistically significant overall differences (pseudo $F_{3,48}$=1.39, P=0.031) (Fig. 6). Pairwise comparisons showed that femoral secretions of lizards in the carotenoid treatment did not differ significantly from control lizards (t=1.10, P=0.19). Secretions of lizards in the carotenoid treatment were significantly different from lizards in the vitamin E treatment (t=1.30, P=0.03) and in the combination of carotenoid and vitamin E treatment (t=1.29, P=0.017). Femoral secretions of lizards in the vitamin E and in the combination of carotenoid and vitamin E treatments were not significantly different (t=1.14, P=0.17), but both were significantly different from the control treatment (t=1.23, P<0.05 for both).

There were also significant differences between treatments in the proportions of vitamin E in femoral secretions (one-way ANOVA on Aitchison's transformed proportions, $F_{3,48}$=52.31, P<0.0001). The femoral secretion in lizards in the vitamin E treatment (55.11±3.72 % vitamin E in secretions) and in the combination of carotenoid and vitamin E treatment (52.55± 4.42 %) did not differ (Tukey's test, P=0.94), and both had significantly higher proportions of vitamin E (P<0.0002 in all cases) than control lizards (12.02±2.78 %) or lizards in the carotenoid treatment (15.60±1.40 %). No significant differences were observed among the two latter groups (P=0.99).

Relationships between visual and chemical signals

When relating proportions of vitamin E in femoral secretions with characteristics of coloration, lizards with higher proportions of vitamin E in secretions had significantly

brighter throats (thPC1; r=−0.31, $F_{1,47}$=5.11, P=0.028) and significantly more saturated "yellowish" (i.e., 500–700 nm) chest coloration (chPC2; r=−0.41, $F_{1,47}$=9.61, P=0.003). Other correlations between vitamin E and the rest of PCs for colorations were not significant (−0.24<r<0.19, P>0.10 in all cases).

Discussion

The results of our experimental supplementation indicated that both carotenoids and vitamin E in the diet affected the expression of visual and chemical signals in *L. schreiberi* lizards. However, different traits were differentially affected, and in most cases, the addition of vitamin E had a higher effect on the expression of ornaments than the addition of carotenoids alone, even for those ornaments that are carotenoid-dependent.

The UV and blue throat coloration of male *L. schreiberi* is considered to be a structural coloration produced by microscopically structured surfaces that interfere with light (Prum 2006; Martín and López 2009; Bajer et al. 2012), which is not carotenoid-dependent.

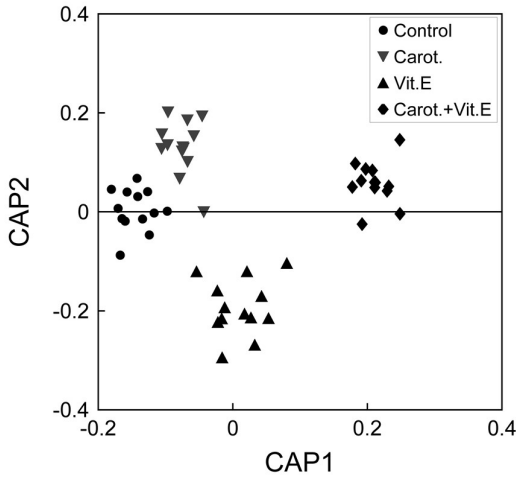

Fig. 6 Representation of the first two axes of the canonical analysis of principal coordinates (CAP) showing classification of the femoral secretions of male lizards *L. schreiberi* at the end of each experimental treatment.

However, we found that both carotenoids alone and vitamin E alone increased brightness compared with control lizards, but that the brightest throat coloration was observed in males supplemented with a combination of carotenoids and vitamin E. However, the chromatic characteristics of throat coloration (i.e., hue and saturation) were not significantly affected by the supplementation. In the closely related *L. viridis*, males with higher UV chroma win more fights, and females prefer males with high throat UV brightness and chroma (Bajer et al. 2010, 2011), and in *L. schreiberi*, dominant males have brighter and more saturated UV and "bluish" throat coloration but have lower immune responses (Martín and López 2009). In male *L. viridis*, a negative trend between body condition and UV throat chroma and between throat brightness/blue throat saturation and ectoparasite load have been observed (Václav et al. 2007; Molnar et al. 2012). These results suggest that it may be costly to produce and maintain bright throat coloration (Bajer et al. 2012). Environmental stress may perturb the biochemical reactions that produce the light scattering structures responsible of structural colors (Kemp and Rutowski 2007; Mäthger and Hanlon 2007). Therefore, males with more antioxidants, such as carotenoids and vitamin E, may experience less physiological stress, allowing them to produce and/or maintain the regularity and spacing of structural components within iridophores, resulting in increased throat brightness. In fact, iridophores account for chromatic variation of carotenoid-dependent coloration in *L. vivipara*, which may be mediated by the nonpigmentary vitamin A_1 (San José et al. 2013).

Chest coloration of *L. schreiberi* is a typical carotenoid-dependent trait (Martín and López 2009). In our experiment, supplemented lizards had more saturated "yellowish" chests than control lizards, but lizards supplemented with vitamin E had more saturated chest "yellowish" coloration than lizards supplemented with carotenoids alone. Nevertheless, it is possible that we did not provide lizards with sufficient amounts of carotenoids, or of the required specific carotenoids, needed to increase "yellowish" coloration. Surprisingly, the chest coloration of males provided with a combination of carotenoids and vitamin E was lower than when only vitamin E was supplemented. We would have expected an additive effect because these two antioxidants have synergistic effects in vivo (Leibovitz et al. 1990;

Palozza and Krinsky 1992). However, supplemented carotenoids might have impaired the intestinal absorption of vitamin E because of competition for the same intestinal membrane transporter, as reported in humans (Reboul et al. 2007).

In summary, our results support the hypothesis that nonpigmentary antioxidants, such as vitamin E, are needed in addition to carotenoids to increase the expression of coloration of male *L. schreiberi*, which confirms the conclusions of other studies testing this hypothesis in other animals (Bertrand et al. 2006; Pike et al. 2007; Pérez et al. 2008). Our results could not, however, discriminate between two possible explanations for this result that is (1) the supplementation of vitamin E might be required to specifically diminish the oxidation of carotenoids used in coloration (Hartley and Kennedy 2004), or (2) supplemented vitamin E might be used as a general antioxidant, which would then allow use of carotenoids for coloration rather than for antioxidant functions (von Schantz et al. 1999; Blount et al. 2003).

In contrast to the effects of the dietary supplementation on male coloration of the chest and throat, the supplementation of vitamin E or carotenoids, alone or in combination, did not affect the dorsal green coloration of male *L. schreiberi*. Green coloration might require some additional pigments or follow alternative physiological pathways. Thus, similarly to our results, green plumage coloration of greenfinches was not affected by vitamin E supplementation (Karu et al. 2008). Alternatively, lizards might not increase green dorsal coloration, even if adequate amount of carotenoids and vitamin E are available, as brighter green coloration may expose the males to an increased risk of predation (Martín and López 2001).

The chemical profile of femoral secretions also varied between treatments; lizards supplemented with vitamin E alone or combined with carotenoids had different chemical profiles than lizards supplemented with carotenoids alone and control lizards. A similar change in femoral secretions was found in *L. viridis* supplemented with vitamin E (Kopena et al. 2011). These results support the hypothesis that an increase in dietary nonpigmentary antioxidants will be directly reflected in the femoral secretions. Because female *L. viridis*

seem to be attracted to areas scent-marked by males with more vitamin E in secretions, it was suggested that this compound may act as an honest signal of male quality (Kopena et al. 2011). Vitamin E in secretions may signal the ability to obtain sufficient antioxidants, such that only males of high quality could allocate large amounts of vitamin E to the femoral secretions without causing a concomitant reduction in their antioxidant capacity (Brigelius-Flohe and Traber 1999; Martín and López 2010; Kopena et al. 2011).

Interestingly, there was a concordance between characteristics of visual and chemical signals; lizards with higher proportions of vitamin E in secretions also had brighter throats and more saturated "yellowish" chests. A similar concordance between signals of multiple types was found in *L. lepida* (Martín and López 2010) and in *Podarcis muralis* lizards (Martín et al. 2008). This supports the idea that visual and chemical signals may be used in different contexts conveying similar messages in different sensory channels.

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Ethical standards

Captures of lizards and experiments complied with all current laws of Spain and were performed under license (permit number: 10/142790.9/11) from the Environmental Agency of Madrid Government ("Consejería del Medio Ambiente de la Comunidad de Madrid", Spain).

References

Aitchison J (1986) The statistical analysis of compositional data. Chapman and Hall, London

Allen DG, Dowling PM, Smith DA, Pasloske K, Woods JP (2004) Handbook of veterinary drugs, 3rd edn. Lippincott Williams and Wilkins, Hoboken

Alonso-Alvarez C, Bertrand S, Devevey G, Gaillard M, Prost J, Faivre B, Sorci G (2004) An experimental test of the dose dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. Am Nat 164:651–659

Anderholm S, Olsson M, Wapstra E, Ryberg K (2004) Fit and fat from enlarged badges: a field experiment on male sand lizards. Proc R Soc Lond B (Suppl) 271:142–144

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Austr Ecol 26:32–46

Anderson MJ, Willis TJ (2003) Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. Ecology 84:511–525

Anderson MJ, Gorley RN, Clarke KR (2008) PERMANOVA+ for PRIMER: guide to software and statistical methods. PRIMER-E Ltd, Plymouth

Andersson M (1994) Sexual selection. Princeton University Press, Princeton

Bajer K, Molnár OR, Török J, Herczeg G (2010) Female European green lizards (*Lacerta viridis*) prefer males with high ultraviolet throat reflectance. Behav Ecol Sociobiol 64:2007–2014

Bajer K, Molnár OR, Török J, Herczeg G (2011) Ultraviolet nuptial colour determines fight success in male European green lizards (*Lacerta viridis*). Biol Lett 7:866–868

Bajer K, Molnár OR, Török J, Herczeg G (2012) Temperature, but not available energy, affects the expression of a sexually selected ultraviolet (UV) colour trait in male European green lizards. PLoS One 7: e34359

Bender DA (2009) Nutritional biochemistry of the vitamins, 2nd edn. Cambridge University Press, Cambridge

Bertrand S, Faivre B, Sorci G (2006) Do carotenoid-based sexual traits signal the availability of non-pigmentary antioxidants? J Exp Biol 209:4414–4419

Blount JD, Metcalfe NB, Birkhead TR, Surai PF (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300:125–127

Brigelius-Flohe R, Traber MG (1999) Vitamin E: function and metabolism. FASEB J 13:1145–1155

Burton GW, Traber MG (1990) Vitamin E: antioxidant activity, biokinetics, and bioavailability. Ann Rev Nutr 10:357–382

Carazo P, Font E, Desfilis E (2007) Chemosensory assessment of rival competitive ability and scent mark function in a lizard (*Podarcis hispanica*). Anim Behav 74:895–902

Clarke KR, Gorley RN (2006) PRIMER v6: user manual/tutorial. PRIMER-E Ltd, Plymouth

Costantini D, Fanfani A, Dell'Omo G (2007) Carotenoid availability does not limit the capability of nestling kestrels (*Falco tinnunculus*) to cope with oxidative stress. J Exp Biol 210:1238–1244

Cooper WE, Greenberg N (1992) Reptilian coloration and behavior. In: Gans C, Crews D (eds) Biology of the Reptilia, vol 18. University of Chicago Press, Chicago, pp 298–422

Cuthill IC, Bennett ATD, Partridge JC, Maier EJ (1999) Plumage reflectance and the objective assessment of avian sexual dichromatism. Am Nat 153:183–200

Endler JA (1990) On the measurement and classification of color in studies of animal color patterns. Biol J Linn Soc 41:315–352

Faivre B, Grégoire A, Préault M, Cézilly F, Sorci G (2003) Immune activation rapidly mirrored in a secondary sexual trait. Science 300: 103

Fitze PS, Cote J, San-Jose LM, Meylan S, Isaksson C, Andersson S, Rossi J-M, Clobert J (2009) Carotenoid-based colours reflect the stress response in the common lizard. PLoS One 4:e5111

Fleishman LJ, Loew ER, Leal M (1993) Ultraviolet vision in lizards. Nature 365:397

Grill CP, Rush VN (2000) Analysing spectral data: comparison and application of two techniques. Biol J Linn Soc 69:121–138

Hamilton PS, Sullivan BK (2005) Female mate attraction in ornate tree lizards, *Urosaurus omatus*: a multivariate analysis. Anim Behav 69: 219–224

Hartley RC, Kennedy MW (2004) Are carotenoids a red herring in sexual display? Trends Ecol Evol 19:353–354

Hill GE, Johnson JD (2012) The vitamin A-redox hypothesis: a biochemical basis for honest signaling via carotenoid pigmentation. Am Nat 180:E127–E150

Isaksson C, Mclaughlin P, Monaghan P, Andersson S (2007) Carotenoid pigmentation does not reflect total non-enzymatic antioxidant activity in plasma of adult and nestling great tits, *Parus major*. Funct Ecol 21:1123–1129

Johnsen A, Delhey K, Andersson SA, Kempenaers B (2003) Plumage color in nestling blue tits: sexual dichromatism, condition dependence and genetic effects. Proc R Soc Lond B 270:1263–1270

Karu U, Saks L, Hörak P (2008) Carotenoid-based plumage coloration is not affected by vitamin E supplementation in male greenfinches. Ecol Res 23:931–935

Kemp DJ, Rutowski RL (2007) Condition dependence, quantitative genetics, and the potential signal content of iridescent ultraviolet butterfly coloration. Evolution 61:168–183

Kopena R, López P, Martín J (2009) Lipophilic compounds from the femoral gland secretions of male Hungarian green lizards, *Lacerta viridis*. Z Naturforsch C 64:434–440

Kopena R, Martín J, López P, Herczeg G (2011) Vitamin E supplementation increases the attractiveness of males' scent for female European green lizards. PLoS One 6:e19410

Kwiatkowski MA, Sullivan BK (2002) Geographic variation in sexual selection among populations of an Iguanid lizard, *Sauromalus obesus* (=*ater*). Evolution 56:2039–3051

Leibovitz B, Hu ML, Tappel L (1990) Dietary supplements of vitamin E, β-carotene, coenzyme Q_{10} and selenium protect tissues against lipidperoxidation in rat tissue slices. J Nutr 120:97–104

Loew ER, Fleishman LJ, Foster RG, Provencio I (2002) Visual pigments and oil droplets in diurnal lizards: a comparative study of Caribbean anoles. J Exp Biol 205:927–938

López P, Martín J (2002) Chemical rival recognition decreases aggression levels in male Iberian wall lizards, *Podarcis hispanica*. Behav Ecol Sociobiol 51:461–465

López P, Martín J (2006) Lipids in the femoral gland secretions of male Schreiber's green lizards, *Lacerta schreiberi.* Z Naturforsch C 61: 763–768

López P, Martín J (2011) Male Iberian rock lizards may reduce the costs of fighting by scent-matching of the resource holders. Behav Ecol Sociobiol 65:1891–1898

López P, Amo L, Martín J (2006) Reliable signaling by chemical cues of male traits and health state in male lizards, *Lacerta monticola*. J Chem Ecol 32:473–488

López P, Gabirot M, Martín J (2009) Immune challenge affects sexual coloration of male Iberian wall lizards. J Exp Zool A 311:96–104

Mader DR (1996) Reptile medicine and surgery. WB Saunders, Philadelphia

Martín J, Forsman A (1999) Social costs and development of nuptial coloration in male *Psammodromus algirus* lizards: an experiment. Behav Ecol 10:396–400

Martín J, López P (2000) Chemoreception, symmetry and mate choice in lizards. Proc R Soc Lond B 267:1265–1269

Martín J, López P (2001) Predation risk may explain the absence of nuptial coloration in the wall lizard, *Podarcis muralis*. Evol Ecol Res 3:889–898

Martín J, López P (2006a) Links between male quality, male chemical signals, and female mate choice in Iberian Rock Lizards. Funct Ecol 20:1087–1096

Martín J, López P (2006b) Vitamin D supplementation increases the attractiveness of males' scent for female Iberian rock lizards. Proc R Soc Lond B 273:2619–2624

Martín J, López P (2007) Scent may signal fighting ability in male Iberian rock lizards. Biol Lett 3:125–127

Martín J, López P (2009) Multiple color signals may reveal multiple messages in male Schreiber's green lizards, *Lacerta schreiberi*. Behav Ecol Sociobiol 63:1743–1755

Martín J, López P (2010) Multimodal sexual signals in male ocellated lizards *Lacerta lepida*: vitamin E in scent and green coloration may signal male quality in different sensory channels. Naturwissenschaften 97:545–553

Martín J, López P (2011) Pheromones and reproduction in Reptiles. In: Norris DO, Lopez KH (eds) Hormones and reproduction of vertebrates. Reptiles, vol 3. Academic Press, San Diego, pp 141–167

Martín J, Civantos E, Amo L, López P (2007) Chemical ornaments of male lizards *Psammodromus algirus* may reveal their parasite load and health state to females. Behav Ecol Sociobiol 62:173–179

Martín J, Amo L, López P (2008) Parasites and health affect multiple sexual signals in male common wall lizards, *Podarcis muralis*. Naturwissenschaften 95:293–300

Martínez A, Rodríguez-Girones MA, Barbosa A, Costas M (2008) Donator acceptor map for carotenoids, melatonin and vitamins. J Phys Chem A 112:9037–9042

Mason RT (1992) Reptilian pheromones. In: Gans C, Crews D (eds) Biology of the Reptilia, vol 18. University of Chicago Press, Chicago, pp 114–228

Mason RT, Parker MR (2010) Social behavior and pheromonal communication in reptiles. J Comp Phys A 196:729–749

Mäthger LM, Hanlon RT (2007) Malleable skin coloration in cephalopods: selective reflectance, transmission and absorbance of light by chromatophores and iridophores. Cell Tissue Res 329:179–186

McArdle BH, Anderson MJ (2001) Fitting multivariate models to community data: a comment on distance-based redundancy analysis. Ecology 82:290–297

McGraw KJ, Ardia DR (2003) Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. Am Nat 162:704–712

Møller AP, Biard C, Blount JD, Houston DC, Ninni P, Saino N, Surai PF (2000) Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? Avian Poult Biol Rev 11:137–159

Molnár O, Bajer K, Török J, Herczeg G (2012) Individual quality and nuptial throat colour in male European green lizards. J Zool 287: 233–239

Montgomerie R (2006) Analyzing colors. In: Hill GE, McGraw KJ (eds) Bird coloration vol 1. Mechanisms and measurements. Harvard University Press, Cambridge, pp 90–147

Olson VA, Owens IPF (1998) Costly sexual signals: are carotenoids rare, risky or required? Trends Ecol Evol 13:510–514

Olsson M (1994) Why are sand lizard males (*Lacerta agilis*) not equally green? Behav Ecol Sociobiol 35:169–173

Olsson M, Madsen T, Nordby J, Wapstra E, Ujvari B, Wittsell H (2003) Major histocompatibility complex and mate choice in sand lizards. Proc R Soc Lond B (Suppl) 270:S254–S256

Olsson M, Wilson M, Isaksson C, Uller T, Mott B (2008) Carotenoid intake does not mediate a relationship between reactive oxygen species and bright colouration: experimental test in a lizard. J Exp Biol 211:1257–1261

Palozza P, Krinsky NI (1992) β-Carotene and α-tocopherol are synergistic antioxidants. Arch Biochem Biophys 297:184–187

Pérez C, Lores M, Velando A (2008) Availability of nonpigmentary antioxidant affects red coloration in gulls. Behav Ecol 19:967–973

Pérez-Rodríguez L (2009) Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. Bioessays 31:1116–1126

Peters A, Denk AG, Delhey K, Kempenaers B (2004) Carotenoid-based bill colour as an indicator of immunocompetence and sperm performance in male mallards. J Evol Biol 17:1111–1120

Pike TW, Blount JD, Lindström J, Metcalfe NB (2007) Availability of non-carotenoid antioxidants affects the expression of a carotenoid-based sexual ornament. Biol Lett 3:353–356

Prum RO (2006) Anatomy, physics, and evolution of structural colors. In: Hill GE, McGraw KJ (eds) Bird coloration vol 1. Mechanisms and measurements. Harvard University Press, Cambridge, pp 295–353

Reboul E, Thap S, Perrot E, Amiot MJ, Lairon D, Borel P (2007) Effect of the main dietary antioxidants (carotenoids, γ-tocopherol, polyphenols, and vitamin C) on α-tocopherol absorption. Eur J Clinic Nutr 61:1167–1173

Salvador A, Díaz JA, Veiga JP, Bloor P, Brown RP (2008) Correlates of reproductive success in male lizards of the alpine species *Iberolacerta cyreni*. Behav Ecol 19:169–176

San-Jose LM, Granado-Lorencio F, Fitze PS (2012) Dietary lipids reduce the expression of carotenoid-based coloration in *Lacerta vivipara*. Funct Ecol 26:646–656

San-Jose LM, Granado-Lorencio F, Sinervo B, Fitze PS (2013) Iridophores and not carotenoids account for chromatic variation of carotenoid-based coloration in common lizards (*Lacerta vivipara*). Am Nat 181:396–409

Simons MJP, Cohen AA, Verhulst S (2012) What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress state in birds—a meta-analysis. PLoS One 7:e43088

Sokal RR, Rohlf FJ (1995) Biometry, 3rd edn. WH Freeman, New York

Steffen JE, Hill GE, Guyer C (2010) Carotenoid access, nutritional stress, and the dewlap color of male brown anoles. Copeia 2010:239–246

Svensson PA, Wong BBM (2011) Carotenoid-based signals in behavioural ecology: a review. Behaviour 148:131–189

Thompson CW, Moore MC (1991) Throat colour reliably signals status in male tree lizards *Urosaurus ornatus*. Anim Behav 42:298–442

Václav R, Prokop P, Fekiac V (2007) Expression of breeding coloration in European green lizards (*Lacerta viridis*): variation with morphology and tick infestation. Can J Zool 85:1199–1206

von Schantz T, Bensch S, Grahn M, Hasselquist D, Wittzell H (1999) Good genes, oxidative stress and condition-dependent sexual signals. Proc R Soc Lond B 266:1–12

Woodall AA, Lee SW, Weesie RJ, Jackson MJ, Britton G (1997) Oxidation of carotenoids by free radicals: relationship between structure and reactivity. Bioch Biophys Acta 1336:33–42

Whiting MJ, Stuart-Fox D, O'Connor D, Firth D, Bennett NC, Blomberg SP (2006) Ultraviolet signals ultra-aggression in a lizard. Anim Behav 72:353–363

Chapter III

This chapter reproduces entirely the manuscript:

Kopena R, López P, Martín J (2014) What are carotenoids signaling? Immunostimulatory effects of dietary vitamin E, but not of carotenoids, in Iberian green lizards. Naturwissenschaften 101:1107-1114

What are carotenoids signaling? Immunostimulatory effects of dietary vitamin E, but not of carotenoids, in Iberian green lizards

Renata Kopena, Pilar López and José Martín

Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain

Abstract

In spite that carotenoid-based sexual ornaments are one of the most popular research topics in sexual selection of animals, the antioxidant and immunostimulatory role of carotenoids, presumably signaled by these colorful ornaments, is still controversial. It has been suggested that the function of carotenoids might not be as an antioxidant per se, but that colorful carotenoids may indirectly reflect the levels of nonpigmentary antioxidants, such as melatonin or vitamin E. We experimentally fed male Iberian green lizards (*Lacerta schreiberi*) additional carotenoids or vitamin E alone, or a combination of carotenoids and vitamin E dissolved in soybean oil, whereas a control group only received soybean oil. We examined the effects of the dietary supplementations on phytohaemagglutinin (PHA)-induced skin-swelling immune response and body condition. Lizards that were supplemented with vitamin E alone or a combination of vitamin E and carotenoids had greater immune responses than control lizards, but animals supplemented with carotenoids alone had lower immune responses than lizards supplemented with vitamin E and did not differ from control lizards. These results support the hypothesis that carotenoids in green

lizards are not effective as immunostimulants, but that they may be visually signaling the immunostimulatory effects of non-pigmentary vitamin E. In contrast, lizards supplemented with carotenoids alone have higher body condition gains than lizards in the other experimental groups, suggesting that carotenoids may be still important to improve condition.

Keywords: Body condition · Carotenoids · Immunostimulatory effects · *Lacerta schreiberi* · Lizard · Sexual ornaments · Tocopherol

Introduction

The evolution of carotenoid-based sexual ornaments has been one of the most popular topics in behavioral and evolutionary ecology in the last 25 years, and several hypotheses have been suggested about the signaling role of carotenoids (reviewed in Olson and Owens 1998; Pérez-Rodríguez 2009; Svensson and Wong 2011). Carotenoids and related metabolites have several important roles in the body such as photoprotection of the retina and the skin (Thomson et al. 2002a, b; Vorobyev 2003; Roberts et al. 2009), a wide range of gene activation and regulatory processes of regeneration, morphogenesis, development, and hormone production (Garbe et al. 1992; Stephensen et al. 2002; Geissmann et al. 2003). Animals are not able to synthesize de novo carotenoid pigments (Goodwin 1984), which have to be acquired from the diet. Carotenoids have antioxidant effects in vitro and may be effective antioxidants in the organism too (Pérez-Rodríguez 2009; Simons et al. 2012). The antioxidant system has very strong relationships with the immune system because macrophages (B and T lymphocytes) and neutrophils produce reactive oxygen species (ROS) when responding to an antigen (Halliwell and Gutteridge 2007) and carotenoids may enhance the immune response by ROS scavenging (Burton 1989; Chew and Park 2004). Thus, carotenoids may have an immunostimulatory effect because of their

antioxidant role (von Schantz et al. 1999; Faivre et al. 2003). Therefore, there may be a trade-off between maintaining the antioxidant system and the immune response and elaborating colorful sexual ornaments (von Schantz et al. 1999). This would allow carotenoid-based sexual ornaments to be honest signals of health and condition (Lozano 1994; McGraw 2006).

Nevertheless, an alternative hypothesis (Hartley and Kennedy 2004) suggests that the biological role of carotenoids might not be as antioxidants per se, but that colorful carotenoids in sexual signals would just reflect and provide "information" on the true organismal antioxidants, such as the level of melatonin, vitamin C, or vitamin E (Burton and Traber 1990; Brigelius-Flohe and Traber 1999; Martínez et al. 2008), which are, however, not visually informative because they are colorless. This may be explained because oxidation of carotenoids alters or destroys their color (Woodall et al. 1997). Therefore, when a sexual ornament shows carotenoids with their color intensity preserved, it would indicate that there are also other types of efficient colorless antioxidants needed for the protection of color of carotenoids (Hartley and Kennedy 2004). Nevertheless, carotenoids not only have effects on the immune system as antioxidants. They can be involved in the activation of thymocytes (Garbe et al. 1992), the expression of immune-related genes (Geissmann et al. 2003) and the up-regulation of proteins involved in cell-to-cell communication (Basu et al. 2001) and they can increase membrane fluidity (Chew and Park 2004). Therefore, there still may be a trade-off between allocating carotenoids to ornament pigmentation or to the immune response.

There are controversial results on the immunostimulatory effect of carotenoids; several studies have found a positive relationship between carotenoids and some aspects of the immune response in birds (Blount et al. 2003; McGraw and Ardia 2003; McGraw et al. 2006; Aguilera and Amat 2007; Stirnemann et al. 2010) and fish (Amar et al. 2000, 2001, 2004; Clotfelter et al. 2007), but there are also many examples demonstrating a lack of association both in birds (Navara and Hill 2003; McGraw and Ardia 2005; Biard et al. 2006; Hõrak et al. 2006; McGraw and Klasing 2006; McGraw et al. 2006; Sutherland et al. 2012) and fish (Lin et al. 2010). A meta-analysis shows a low effect size for the relationships of circulating carotenoid level with immunocompetence and oxidative stress in birds,

suggesting that these relationships might not be causal and that complementary mechanisms maintaining honesty might be involved (Simons et al. 2012). However, studies on the role of carotenoids in other animals also showing carotenoid-dependent coloration, such as some lizards, are less frequent (Olsson et al. 2008; Fitze et al. 2009; López et al. 2009; San-José et al. 2012a, b; Kopena et al. 2014; McCartney et al. 2014), and the relationship between carotenoid intake and immune response has been little explored.

In this study, we examined in the Schreiber's green lizard (*Lacerta schreiberi*) the immunostimulatory effects of dietary carotenoids in comparison with the effects of a strong, also dietary and lipid-soluble antioxidant, the vitamin E. This lizard is a large sexually dimorphic lacertid from the Iberian Peninsula. Males have, especially during the breeding season, green dorsal coloration with small black spots, yellow chest, and ventral coloration, and bright blue and UV throat and mental coloration (Martín and López 2009). In contrast, females are mainly dull brown, with pale yellow vents. Interindividual variation in the characteristics of males' breeding coloration may signal variation in morphology, health state, dominance, and pairing status (Martín and López 2009). In a previous study, we found that both carotenoids and vitamin E in the diet affect the expression of color ornaments of male *L. schreiberi* lizards. However, in most cases, the addition of vitamin E has a higher effect on the expression of coloration of ornaments than the addition of carotenoids alone, even for those ornaments that are not carotenoid dependent (Kopena et al. 2014).

Here, we experimentally fed male lizards *L. schreiberi* supplementary carotenoids or vitamin E alone, or a combination of carotenoids and vitamin E, dissolved in soybean oil, whereas a control group only received soybean oil. Thereafter, we measured the phytohaemagglutinin (PHA)-induced immune response and the body condition of lizards. We expected that if carotenoids had immunostimulatory effects, lizards supplemented with carotenoids alone would have higher skin-swelling immune responses than control lizards. Moreover, lizards supplemented with vitamin E (alone or combined) would have higher immune responses than control lizards due to the radical scavenger effect of vitamin E (Burton and Traber 1990; Brigelius-Flohe and Traber 1999). Finally, when carotenoids and

vitamin E were combined, these would have synergistic effects increasing the immune response. However, if the Hartley and Kennedy's (2004) hypothesis was true and carotenoids were not true antioxidants in vivo, the immune responses in the carotenoid treatment would not differ from the control one, and the immune responses in the vitamin E and in the combined treatments would be similarly high.

Materials and methods

Study animals

In April 2011, we collected 48 adult male lizards L. schreiberi from a population inhabiting a pine forest that occupy two contiguous small valleys ("Valle de La Fuenfría" and "Valle de Navalmedio") (40°44 N, 4°02 W; Cercedilla, Madrid Province, Spain) in the Guadarrama mountains. We immediately transferred lizards to "El Ventorrillo" field station of the Museo Nacional de Ciencias Naturales (Madrid province, Spain), 5 km from the capture site. During all the experiment, lizards were individually kept in outdoor 51×36×28 cm PVC terraria containing coconut fiber substratum and rocks for cover. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*), house crickets (*Acheta domesticus*), and common black crickets (*Gryllus assimilis*) dusted with calcium powder, and water was provided ad libitum. At the end of the study, all lizards were released at their exact capture sites in good condition.

We measured immediately after capture and again at the end of the experiment males' body weight with a digital balance to the nearest 0.1 g (initial weight: X ±SE=26.5± 0.7 g; range=18.0–40.5 g) and used a ruler to measure snout-to-vent length, SVL to the nearest 1 mm (initial SVL: X ± SE=98±1 mm; range=84–110 mm) and tail length (X ±SE= 157±5 mm; range=53–204 mm). Individual values of "body condition" were calculated as the residuals from the regression equation of ln mass (in grams) on ln SVL (in millimeter), which may represent an index of the relative amount of fat stored and hence an estimation

of individual physical condition or nutritional status (Bonnet and Naulleau 1994). Only males (N=46) with complete or entirely regenerated tails were considered to estimate body condition.

Experimental supplementation of the diet

We selected randomly which individuals were assigned to each of four treatments ("carotenoids", "vitamin E", "combination of vitamin E and carotenoids" or "control"; see below). Each male was subjected to daily supplementation for 28 days in his own individual terrarium.

In the "carotenoid" treatment, male lizards were fed daily 8 μg of a mix of a carotenoid complex (Solgar Natural Lutein Lycopene Carotene Complex, purchased from Solgar Inc., Leonia, New Jersey, USA), which contained β-carotene, α-carotene, zeaxanthin, lutein, and lycopene (in a rate of 5:1.7:0.3:5:5), dissolved in 20 μL soybean oil. Thus, after 28 days of daily supplementation, each male had been provided with a total of 224 μg of carotenoids. The daily amount of carotenoids provided was based on previous studies of carotenoid supplementation in other lizards (Olsson et al. 2008) and corrected for differences in body size between species. Although there is no information on the types of carotenoids found in L. schreiberi, studies of another lacertid lizard species reported that lutein and zeaxanthin were the most abundant carotenoids in all tisues, followed by low concentrations of β-carotene (Czeczuga 1980; Fitze et al. 2009; San-José et al. 2012b), all of which were available in our supplementation. This carotenoid profile could result directly from the insectivorous diets of these lizards (e.g., Isaksson and Andersson 2007).

In the "vitamin E" treatment, males were fed daily 20 μL vitamin E supplement (synthetic (±)-α-tocopherol; purchased from Sigma-Aldrich Chemicals Co., St. Louis, Missouri, USA). This contained 97 % of synthetic vitamin E (approximately 1,014 IU mL^{-1}) and 3 % soybean oil (with approximately 0.32 IU mL^{-1} of natural vitamin E, i.e., D-α-tocopherol). Thus, the daily dose provided for each male was approximately 20.2 IU of

vitamin E, which is close to the daily minimal physiological necessity of vitamin E for similar-sized reptiles, and well below the tolerable upper intake levels (Mader 1996; Allen et al. 2004; Bender 2009). In other lacertid lizard species, α-tocopherol was found in abundance in all tissues (San-José et al. 2012b). In the "combination of carotenoids and vitamin E" treatment, male lizards were fed daily 8 µg of carotenoids and 20 µL of vitamin E as above, both mixed and dissolved together in 20 µL soybean oil. Finally, in the "control" treatment, males were fed daily 20 µL soybean oil alone. To ensure that all lizards ingested the same amount of carotenoid or vitamin E supplement or the control solution, we gently handled lizards and used sterile plastic syringes with a canula to slowly deliver the solution into their mouth, thus ensuring that lizards swallowed the entire dose.

Immune response

One of the most widespread methods for measuring in vivo the immune response is the phytohaemagglutinin (PHA) skin-swelling test (Smits et al. 1999; Kennedy and Nager 2006; de Bellocq et al. 2007; Ardia and Schat 2008). PHA is a plant lectin that induces an artificial activation of the immune system. Although, this test was first referred as an indicator of T-cell-mediated immunocompetence (Hawley et al. 2009; Kilgas et al. 2010), recent studies suggest that the reaction to the PHA injection might be a nonspecific complex inflammation connected with massive infiltration of cells representing both adaptive and innate immunity (Martin et al. 2006: Sarv and Hõrak 2009; Vinkler et al. 2010, 2012; Salaberria et al. 2013). Therefore, the PHA-induced swelling may be a multifaceted index of cutaneous immune activity, and we used this test because we were interested in a standardized index of immunocompetence (Salaberria et al. 2013), independent of the type of immune cells involved.

One day after finishing the diet supplementation procedure, we measured the immune response of lizards by using the PHA injection test (Smits et al. 1999). We used pressure-sensitive spessimeter to measure thickness (to the nearest 0.01 mm) at the same point of the right hindlimb foot pad before and 24 h after injecting 0.04 mg of PHA dissolved

in 0.02 ml of phosphate-buffered saline (PBS) at the marked point. We calculated the immune response as the difference between pre- and postinjection thickness measures (Smits et al. 1999). The only appreciable effect of the PHA injection was a slight swelling of the skin, due to the immune response, which disappeared after 48 h. No lizard showed any sign of stress or pain due to this test.

Results

There were significant differences among treatments in the magnitude (log-transformed) of the PHA-induced immune response of lizards after the diet supplementation (one-way ANOVA, $F_{3,44}$=5.74, P=0.002, Fig. 1). Post hoc tests showed that responses of control lizards did not differ significantly from those of lizards supplemented with carotenoids alone (Tukey's test, P=0.98), but had significantly lower responses than lizards supplemented with vitamin E alone (P<0.018), or with a combination of vitamin E and carotenoids (P<0.01). Lizards supplemented with carotenoids alone had significantly lower responses than lizards supplemented with vitamin E alone (P<0.05), but differences between lizards supplemented with carotenoids alone and lizards supplemented with a combination of vitamin E and carotenoids only approached significance (P=0.078). Lizards supplemented with vitamin E alone did not significantly differ of lizards supplemented with both vitamin E and carotenoids combined (P=0.99).

There were not significant differences among treatments in initial body size (one-way ANOVAs, weight: $F_{3,44}$=0.09, P= 0.97; SVL: $F_{3,44}$=0.10, P=0.96) or initial body condition of lizards ($F_{3,42}$ =0.53, P=0.66). However, the difference between body condition at the end and at the start of the experiment varied significantly among treatments (one-way ANOVA, $F_{3,42}$=3.76, P=0.018). Lizards supplemented with carotenoids alone had positive and significantly greater changes in body condition than lizards supplemented with vitamin E, alone or combined with carotenoids (Tukey's tests, P<0.03 for both) or control lizards

(P<0.05), all of which had average negative changes in body condition that not differ significantly among them (P>0.88 for all).

The PHA-induced immune response was not significantly related with body condition of lizards at the end of the experiment (r=−0.10, $F_{1,44}$=0.53, P=0.51) nor with changes in body condition (r=−0.04, $F_{1,44}$=0.07, P=0.79) or body size (weight, r = −0.15, $F_{1,46}$ = 1.08, P = 0.30; SVL, r =−0.13, $F_{1,46}$=0.83, P=0.37).

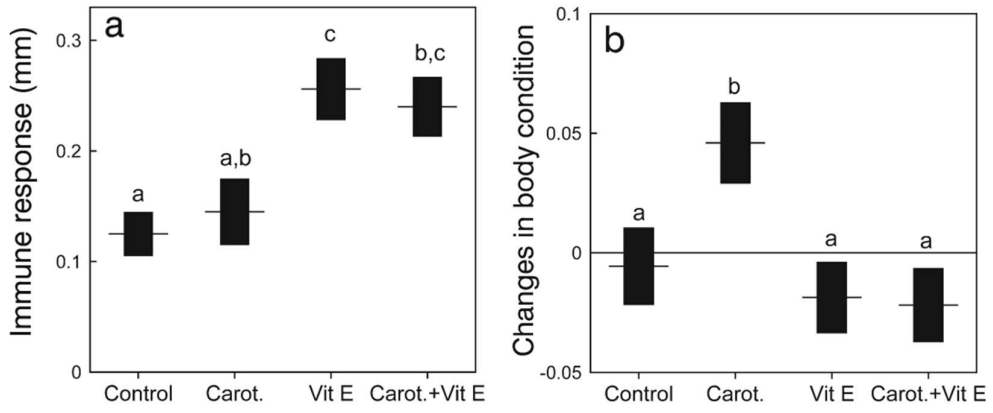

Fig. 1 PHA-induced immune response (X ±1 SE; in millimeter)(**a**) and change in body condition (X ±1 SE)(**b**) of male lizards *L. schreiberi* in each experimental treatment. Means with the same letter above the bars were not significant different.

Discussion

Our results showed that some of the experimental diet supplementations, but not others, affected the immune response of male *L. schreiberi* lizards. Lizards that were supplemented with vitamin E had heightened PHA skin-swelling immune responses, while the supplementation of carotenoids did not seem to have any effect in comparison with control lizards. Therefore, this experiment may support the hypothesis that carotenoids are not effective immunostimulants, at least in this lizard species and with respect to the aspects of the immune response measured by the PHA test.

Contrary to our results, several studies in birds and fish have found a positive effect of dietary carotenoids on the PHA skin-swelling immune response (Blount et al. 2003; McGraw and Ardia 2003; Aguilera and Amat 2007; Clotfelter et al. 2007; Stirnemann et al. 2010), or found that the immune response depletes available carotenoids (McGraw and Ardia 2003; Alonso-Alvarez et al. 2004; Aguilera and Amat 2007; Pérez-Rodríguez et al. 2008) or found a positive relationship between circulating carotenoid levels and the magnitude of the immune response (McGraw and Ardia 2003; Pérez-Rodríguez et al. 2008). However, many other studies of birds and fish show a lack of effects of carotenoid supplementation on the immune response (Navara and Hill 2003; McGraw and Ardia, 2005; Biard et al. 2006; Hõrak et al. 2006, 2007; McGraw and Klasing 2006; McGraw et al. 2006; Lin et al. 2010; Sutherland et al. 2012).

We did not find evidence of any effect of carotenoids on the PHA-induced immune response of green lizards. Similarly, carotenoid supplementation has no effects on several aspects of immune performance of growing juvenile chameleons (*Chamaeleo calyptratus*) (McCartney et al. 2014). In agreement, carotenoid intake in male Australian painted dragon lizards (*Ctenophorus pictus*) does not reduce circulating levels of reactive oxygen species (ROS) or baseline superoxide (bSO), suggesting that carotenoids are inefficient antioxidants in vivo in these lizards. However, the PHA test does not show the whole immune response of the body and therefore we have to narrow our conclusions. Thus, in male society finches, *Lonchura domestica*, supplemented carotenoids do not affect the PHA-induced immune response but increase innate immunity (bacterial-killing activity of whole blood) (McGraw et al. 2006). Similarly, greenfinches, *Carduelis chloris*, with brighter yellow breast feathers show stronger humoral immune response against novel antigen (SRBC) while they do not show any relationship between plumage coloration and the PHA response (Saks et al. 2003). Therefore, carotenoids might still have a role in other aspects of the immune response of green lizards not examined here.

Also, it is possible that just feeding an animal with more antioxidants was not enough to increase directly the antioxidant or immunostimulatory capacity. This is because

the levels of different types of antioxidants are homeostatically regulated in animals. Thus, more dietary antioxidants may lead to down-regulation of, for example, antioxidant enzymes in order to maintain some optimal level of antioxidant activity. Further studies might test this possibility with a two-factor study crossing a dietary antioxidant supplement with an antioxidant challenge, which will raise the actual need for additional dietary antioxidants.

Vitamin E supplementation, however, had a clear effect in the heightened PHA-induced immune response of green lizards. Immunostimulatory effects of vitamin E have been repeatedly demonstrated in poultry (Surai 2002). Also, previous studies have shown that green lizards with higher proportions of vitamin E in femoral secretions, which result from higher dietary intake of this vitamin, have heightened immune responses (López and Martín 2006; Kopena et al. 2009, 2011; Martín and López 2010) and are preferred by females (Kopena et al. 2011). This suggests that the cost of allocating antioxidant vitamin E to secretions may confer reliability to chemical signals of green lizards. In contrast, although vitamin E supplementation enhances growth and condition of nestling barn swallows (*Hirundo rustica*), it does not affect their PHA immune response (de Ayala et al. 2006). Similarly, in greenfinches (*C. chloris*), there is no effect of vitamin E on the PHA reaction. However, this could be explained because birds in all treatments were fed sunflower seeds, which are one of the richest seeds in natural vitamin E content and this could mask the differences between the vitamin E supplemented and the control groups (Hõrak et al. 2007). In a similar study, supplementary vitamin E had no effect on the PHA skin-swelling response of 21-week-old ring-necked pheasants (*Phasianus colchicus*), but there were 13 weeks between the vitamin E dietary supplementation and the PHA test and probably pheasants did not store vitamin E to be used as antioxidant but allocated it into development (Orledge et al. 2012).

Lizards supplemented with carotenoids plus vitamin E combined had greater immune responses than control lizards, but these responses only tended to differ marginally of lizards supplemented with carotenoids alone. This is an interesting result, because these two antioxidants may have synergistic effects (Leibovitz et al. 1990; Palozza and Krinsky

1992; Surai et al. 2001). It is possible that increasing carotenoids intake required using part of the supplemented vitamin E to protect these carotenoids from oxidation in colorful traits, and, thus, not all vitamin E could be used in other antioxidant functions. This would result in that lizards supplemented with vitamin E alone would actually have more vitamin E available to be used in antioxidant functions related to the immune response, leading to greater differences between treatments. Another possible reason is that the mechanism of carotenoid absorption in the intestine was similar to that of vitamin E (Woodall et al. 1996; Surai 2002). Therefore, increased carotenoid supplementation might have induced competitive interactions between carotenoids and vitamin E during absorption, and supplemented carotenoids might have impaired the intestinal absorption of vitamin E, as reported in humans (Reboul et al. 2007).

Finally, body condition of lizards supplemented with carotenoids alone increased, while lizards supplemented with vitamin E or with carotenoids combined with vitamin E had decreased body condition. This suggests that carotenoids may have some important role affecting positively to health state and condition of lizards, possibly as antioxidants. However, changes in body condition were not related to differences in the immune response. In some growing nestlings bird species, carotenoid supplementation may also increase body condition by regulating oxidative stress resulting from rapid growth (Biard et al. 2006), although in other fish and bird species there are no effects (e.g., Pike et al. 2010; Sutherland et al. 2012), probably because natural food might contain sufficient carotenoids to obscure any benefit of carotenoid supplementation. A similar positive effect of carotenoids on body condition might occur in lizards which have continuous growth and, in our captivity experiment, only have access to some prey types that might not contain enough natural carotenoids. However, supplementary carotenoids did not increase body weight of captive common lizards, *Lacerta vivipara* (San-José et al. 2012a). Interestingly, the positive effect of carotenoids disappeared when carotenoids were given together with vitamin E, suggesting that dietary vitamin E and carotenoids may interact with each other in a complicated fashion.

In summary, our previous research examining the effects of antioxidants on sexual signals of *L. schreiberi* (Kopena et al. 2014) and the present results support the hypothesis that carotenoids are not effective as immunostimulants, but that they may be visually signaling the immunostimulatory effects of non-pigmentary vitamin E. However, we have to interpret carefully the results of the PHA test as indicator of immunocompetence (Kennedy and Nager 2006) and for clearer conclusions we need further experiments that examine other aspects of the antioxidant and immune system of lizards. Also, the possible synergistic or competitive effects of carotenoids and vitamin E should be examined.

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Ethical standards

The experiments enforced all the present Spanish laws and were performed under license (permit number: 10/142790.9/11) from the Environmental Organisms of Madrid Community where they were carried out.

Conflict of interest

None

References

Aguilera E, Amat JA (2007) Carotenoids, immune response and the expression of sexual ornaments in male greenfinches (*Carduelis chloris*). Naturwissenschaften 94:895–902

Allen DG, Dowling PM, Smith DA, Pasloske K, Woods JP (2004) Handbook of veterinary drugs, 3rd edn. Lippincott Williams and Wilkins, Hoboken

Alonso-Alvarez C, Bertrand S, Devevey G, Gaillard M, Prost J, Faivre B, Sorci G (2004) An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. Am Nat 164:651–659

Amar EC, Kiron V, Satoh S, Okamoto N, Watanabe T (2000) Effects of dietary β-carotene on the immune response of rainbow trout *Oncorhynchus mykiss*. Fish Sci 66:1068–1075

Amar EC, Kiron V, Satoh S, Watanabe T (2001) Influence of various dietary synthetic carotenoids on bio-defence mechanisms in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquacult Res 32:162–173

Amar EC, Kiron V, Satoh S, Watanabe T (2004) Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. Fish Shellfish Immunol 16:527–537

Ardia DR, Schat KA (2008) Ecoimmunology. In: Davison F, Kaspers B, Schat KA (eds) Avian immunology. Academic Press, London, pp 421–441

Basu HN, Del Vecchio AJ, Flider F, Orthoefer FT (2001) Nutritional and potential disease prevention properties of carotenoids. J Am Oil Chem Soc 78:665–675

Bender DA (2009) Nutritional biochemistry of the vitamins, 2nd edn. Cambridge University Press, Cambridge

Biard C, Surai PF, Møller AP (2006) Carotenoid availability in diet and phenotype of blue and great tit nestlings. J Exp Biol 209:1004–1015

Blount JD, Metcalfe NB, Birkhead TR, Surai PF (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300:125–127

Bonnet X, Naulleau G (1994) A body condition index (BCI) in snakes to study reproduction. C R Acad Sci Ser III Sci Vie 317:34–41

Brigelius-Flohe R, Traber MG (1999) Vitamin E: function and metabolism. FASEB J 13:1145–1155

Burton GW (1989) Antioxidant action of carotenoids. J Nutr 119:109– 111

Burton GW, Traber MG (1990) Vitamin E: antioxidant activity, biokinetics, and bioavailability. Annu Rev Nutr 10:357–382

Chew BP, Park JS (2004) Carotenoid action on the immune response. J Nutr 134:257S–261S

Clotfelter ED, Ardia DR, McGraw KJ (2007) Red fish, blue fish: trade-offs between pigmentation and immunity in *Betta splendens*. Behav Ecol 18:1139–1145

Czeczuga B (1980) Carotenoids in some parts of certain species of lizards. Comp Biochem Physiol B 65:755–757

de Ayala RM, Martinelli R, Saino N (2006) Vitamin E supplementation enhances growth and condition of nestling barn swallows (*Hirundo rustica*). Behav Ecol Sociobiol 60:619–630

de Bellocq JG, Porcherie A, Moulia C, Morand S (2007) Immunocompetence does not correlate with resistance to helminth parasites in house mouse subspecies and their hybrids. Parasitol Res 100:321–328

Faivre B, Grégoire A, Préault M, Cézilly F, Sorci G (2003) Immune activation rapidly mirrored in a secondary sexual trait. Science 300:103

Fitze PS, Cote J, San-Jose LM, Meylan S, Isaksson C, Andersson S, Rossi J-M, Clobert J (2009) Carotenoid-based colours reflect the stress response in the common lizard. PLoS One 4:e5111

Garbe A, Buck J, Hämmerling U (1992) Retinoids are important cofactors in T cell activation. J Exp Med 176:109–117

Geissmann F, Revy P, Brousse N, Lepelletier Y, Folli C, Durandy A, Chambon P, Dy M (2003) Retinoids regulate survival and antigen presentation by immature dendritic cells. J Exp Med 198:623–634

Goodwin TW (1984) The biochemistry of carotenoids. Vol. 2: animals. Chapman and Hall, London

Halliwell B, Gutteridge J (2007) Free radicals in biology and medicine. Oxford University Press, Oxford

Hartley RC, Kennedy MW (2004) Are carotenoids a red herring in sexual display? Trends Ecol Evol 19:353–354

Hawley DM, Hallinger KK, Cristol DA (2009) Compromised immune competence in free-living tree swallows exposed to mercury. Ecotoxicology 18:499–503

Hõrak P, Zilmer M, Saks L, Ots I, Karu U, Zilmer K (2006) Antioxidant protection, carotenoids, and the costs of immune challenge in greenfinches. J Exp Biol 209:4329–4338

Hõrak P, Saks L, Zilmer M, Karu U, Zilmer K (2007) Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. Am Nat 170:625–635

Isaksson C, Andersson S (2007) Carotenoid diet and nestling provisioning in urban and rural great tits *Parus major*. J Avian Biol 38:564–572

Kennedy MW, Nager RG (2006) The perils and prospects of using phytohaemagglutinin in evolutionary ecology. Trends Ecol Evol 21:653–655

Kilgas P, Tilgar V, Kulavee R, Saks L, Hõrak P, Mand R (2010) Antioxidant protection, immune function and growth of nestling great tits *Parus major* in relation to within-brood hierarchy. Comp Biochem Physiol B 157:288–293

Kopena R, López P, Martín J (2009) Lipophilic compounds from the femoral gland secretions of male Hungarian green lizards, *Lacerta viridis*. Z Naturforsch C 64:434–440

Kopena R, Martín J, López P, Herczeg G (2011) Vitamin E supplementation increases the attractiveness of males' scent for female European green lizards. PLoS One 6:e19410

Kopena R, López P, Martín J (2014) Relative contribution of dietary carotenoids and vitamin E to visual and chemical sexual signals of male Iberian green lizards: an experimental test. Behav Ecol Sociobiol 68:571–581

Leibovitz B, Hu ML, Tappel AL (1990) Dietary supplements of vitamin E, β-carotene, coenzyme Q_{10} and selenium protect tissues against lipid peroxidation in rat tissue slices. J Nutr 120:97–104

Lin SM, Nieves-Puigdoller K, Brown AC, McGraw KJ, Clotfelter ED (2010) Testing the carotenoid trade-off hypothesis in the polychromatic midas cichlid, *Amphilophus citrinellus*. Physiol Biochem Zool 83:333–342

López P, Martín J (2006) Lipids in the femoral gland secretions of male Schreiber's green lizards, *Lacerta schreiberi*. Z Naturforsch C 61: 763–768

López P, Gabirot M, Martín J (2009) Immune challenge affects sexual coloration of male Iberian wall lizards. J Exp Zool A 311:96–104

Lozano GA (1994) Carotenoids, parasites, and sexual selection. Oikos 70:309–311

Mader DR (1996) Reptile medicine and surgery. WB Saunders, Philadelphia

Martín J, López P (2009) Multiple color signals may reveal multiple messages in male Schreiber's green lizards, *Lacerta schreiberi*. Behav Ecol Sociobiol 63:1743–1755

Martín J, López P (2010) Multimodal sexual signals in male ocellated lizards *Lacerta lepida*: vitamin E in scent and green coloration may signal male quality indifferent sensory channels. Naturwissenschaften 97:545–553

Martin LB, Han P, Lewittes J, Kuhlman JR, Klasing KC, Wikelski M (2006) Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct Ecol 20:290–299

Martínez A, Rodríguez-Girones MA, Barbosa A, Costas M (2008) Donator acceptor map for carotenoids, melatonin and vitamins. J Phys Chem A 112:9037–9042

McCartney KL, Ligon RA, Butler MW, DeNardo DF, McGraw KJ (2014) The effect of carotenoid supplementation on immune system development in juvenile male veiled chameleons (*Chamaeleo calyptratus*). Front Zool 11:26

McGraw KJ (2006) Mechanics of carotenoid-based coloration. In: Hill GE, McGraw KJ (eds) Bird coloration. Harvard University Press, Cambridge, pp 90–147

McGraw KJ, Ardia DR (2003) Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. Am Nat 162:704–712

McGraw KJ, Ardia DR (2005) Sex differences in carotenoid status and immune performance in zebra finches. Evol Ecol Res 7:251–262

McGraw KJ, Klasing KC (2006) Carotenoids, immunity, and integumentary coloration in red junglefowl (*Gallus gallus*). Auk 123:1161–1171

McGraw KJ, Crino OL, Medina-Jerez W, Nolan PM (2006) Effect of dietary carotenoid supplementation on food intake and immune function in a songbird with no carotenoid coloration. Ethology 112:1209–1216

Navara KJ, Hill GE (2003) Dietary carotenoid pigments and immune function in a songbird with extensive carotenoid-based plumage coloration. Behav Ecol 14:909–916

Olson VA, Owens IPF (1998) Costly sexual signals: are carotenoids rare, risky or required? Trends Ecol Evol 13:510–514

Olsson M, Wilson M, Isaksson C, Uller T, Mott B (2008) Carotenoid intake does not mediate a relationship between reactive oxygen species and bright colouration: experimental test in a lizard. J Exp Biol 211:1257–1261

Orledge JM, Blount JD, Hoodless AN, Pike TW, Royle NJ (2012) Synergistic effects of supplementation of dietary antioxidants during growth on adult phenotype in ring-necked pheasants, *Phasianus colchicus*. Funct Ecol 26:254–264

Palozza P, Krinsky NI (1992) β-Carotene and α-tocopherol are synergistic antioxidants. Arch Biochem Biophys 297:184–187

Pérez-Rodríguez L (2009) Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. Bioessays 31:1116–1126

Pérez-Rodríguez L, Mougeot F, Alonso-Álvarez C, Blas J, Viñuela J, Bortolotti GR (2008) Cell-mediated immune activation rapidly decreases plasma carotenoids but does not affect oxidative stress in red-legged partridges (*Alectoris rufa*). J Exp Biol 211:2155–2161

Pike TW, Blount JD, Lindström J, Metcalfe NB (2010) Dietary carotenoid availability, sexual signalling and functional fertility in sticklebacks. Biol Lett 6:191–193

Reboul E, Thap S, Perrot E, Amiot MJ, Lairon D, Borel P (2007) Effect of the main dietary antioxidants (carotenoids, γ-tocopherol, polyphenols, and vitamin C) on α-tocopherol absorption. Eur J Clin Nutr 61:1167–1173

Roberts RL, Green J, Lewis B (2009) Lutein and zeaxanthin in eye and skin health. Clin Dermatol 27:195–201

Saks L, Ots I, Hõrak P (2003) Carotenoid-based plumage coloration of male greenfinches reflects health and immunocompetence. Oecologia 134:301–307

Salaberria C, Muriel J, de Luna M, Gil D, Puerta M (2013) The PHA test as an indicator of phagocytic activity in a passerine bird. PLoS One 8:e84108

San-José LM, Granado-Lorencio F, Fitze PS (2012a) Dietary lipids reduce the expression of carotenoid-based coloration in *Lacerta vivipara*. Funct Ecol 26:646–656

San-José LM, Granado-Lorencio F, Fitze PS (2012b) Vitamin E, vitamin A, and carotenoids in male common lizard tissues. Herpetologica 68:88–99

Sarv T, Hõrak P (2009) Phytohaemagglutinin injection has a long-lasting effect on immune cells. J Avian Biol 40:569–571

Simons MJP, Cohen AA, Verhulst S (2012) What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress state in birds—a meta-analysis. PLoS One 7:e43088

Smits JE, Bortolotti GR, Tella JL (1999) Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. Funct Ecol 13:567–572

Stephensen CB, Rasooly R, Jiang X, Ceddia MA, Weaver CT, Chandraratna RA, Bucy RP (2002) Vitamin A enhances in vitro Th2 development via retinoid X receptor pathway. J Immunol 168: 4495–4503

Stirnemann I, Johnston G, Rich B, Robertson J, Kleindorfer S (2010) Phytohaemagglutinin (PHA) response and bill-hue wavelength increase with carotenoid supplementation in diamond firetails (*Stagonopleura guttata*). Emu 109:344–351

Surai PF (2002) Natural antioxidants in avian nutrition and reproduction. Nottingham University Press, Nottingham

Surai PF, Speake BK, Sparks NHC (2001) Carotenoids in avian nutrition and embryonic development. 2. Antioxidant properties and discrimination in embryonic tissues. J Poult Sci 38:117–145

Sutherland JL, Thompson CF, Sakaluk SK (2012) No effect of carotenoid supplementation on PHA response or body condition of nestling house wrens. Physiol Biochem Zool 85:21–28

Svensson PA, Wong BBM (2011) Carotenoid-based signals in behavioural ecology: a review. Behaviour 148:131–189

Thomson LR, Toyoda Y, Delori FC, Garnett KM, Wong Z-Y, Nichols CR, Cheng KM, Craft NE, Dorey K (2002a) Long term dietary supplementation with zeaxanthin reduces photoreceptor death in light-damaged Japanese quail. Exp Eye Res 75:529–542

Thomson LR, Toyoda Y, Langner A, Delori FC, Garnett KM, Craft N, Nichols CR, Cheng KM, Dorey CK (2002b) Elevated retinal zeaxanthin and prevention of light-induced photoreceptor cell death in quail. Investig Ophthalmol Vis Sci 43:3538–3549

Vinkler M, Bainová H, Albrecht T (2010) Functional analysis of the skin swelling response to phytohaem-agglutinin. Funct Ecol 24:1081–1086

Vinkler M, Schnitzer J, Munclinger P, Albrecht T (2012) Phytohaemagglutinin skin-swelling test in scarlet rosefinch males: low-quality birds respond more strongly. Anim Behav 83:17–23

von Schantz T, Bensch S, Grahn M, Hasselquist D, Wittzell H (1999) Good genes, oxidative stress and condition-dependent sexual signals. Proc R Soc Lond B 266:1–12

Vorobyev M (2003) Coloured oil droplets enhance colour discrimination. Proc R Soc Lond B 270:1255–1261

Woodall AA, Britton G, Jackson MJ (1996) Dietary supplementation with carotenoids: effects on α-tocopherol levels and susceptibility of tissues to oxidative stress. Br J Nutr 76:307–317

Woodall AA, Lee SW, Weesie RJ, Jackson MJ, Britton G (1997) Oxidation of carotenoids by free radicals: relationship between structure and reactivity. Biochem Biophys Acta 1336:33–42

Chapter IV

This chapter reproduces entirely the manuscript:

Kopena R, López P, Martín J Immune challenge and vitamin E supplementation increase development of melanin-based visual sexual signals of male Iberian green lizards. *Under review in Behavioral ecology and sociobiology*

Immune challenge and vitamin E supplementation increase development of melanin-based visual sexual signals of male Iberian green lizards

Renata Kopena, Pilar López and José Martín

Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC. José Gutiérrez Abascal 2, E-28006 Madrid, Spain

Abstract

For a sexual signal to be honest, it is required a link between the signal and fitness, which is supported by costs of the signal and trade-offs between signal development and essential physiological functions, such as the immune defense or the antioxidant system. We experimentally challenged the immune system of *Lacerta schreiberi* male lizards with a bacterial antigen (lipopolysaccharide; LPS). We explored whether the immune activation influenced melanin- and carotenoid-based visual signals and chemical signals. Furthermore, we examined the interactive effects with a vitamin E dietary supplementation, as this vitamin has important antioxidant functions in an immune challenged situation. Lizards with an immune challenge did not decrease the saturation of carotenoid-based visual signals, and increased melanin-based UV-blue signals (i.e., throat coloration), particularly when they had supplementary vitamin E. Males with an immune challenge might try to maximize their current fitness by increasing throat signal intensity in a situation where long-term expectatives of survival and future reproduction are low. Surprisingly, vitamin E decreased carotenoid-based coloration but increased melanin-based coloration, which is opposite to a previous experiment when climatic conditions in the year of study were different. This might

be explained if females showed flexible mate choice, selecting the type of signals that more reliably indicate male quality under different environmental circumstances. Finally, vitamin E content in chemical signals increased in all males supplemented with this vitamin, independently of the immune challenge, which supports vitamin E as an honest signal of male quality.

Significance statement

The trade-offs between sexual signals and the immune and antioxidant systems, which allow honesty of signals, are little investigated, especially in lower vertebrates. We examined in a lizard with multiple types of signals (carotenoid-, melanin- and structural-based coloration, and chemical signals) the effects of a simulated immune challenge on these signals. Furthermore, we studied the interactions with vitamin E in the diet, as this is an important antioxidant and immunostimulant. Surprisingly, carotenoid-based signals were not influenced by the immune activation, but melanin and structural coloration increased, especially if males had more vitamin E. This suggests that challenged, aparently ill, males might try to maximize their current mating success because their survivorship probabilities were low. Additionally, climatic differences and flexible female choice might explain the different effects of vitamin E observed in different years.

Keywords: Carotenoids · Coloration · Chemical signals · Immune System · Lizards · Melanin

Introduction

For a sexual signal to be honest, it is required a link between the signal and some defining property of fitness, which is usually supported by the cost of the signal (Searcy and Nowicki 2005). This cost can be manifested in several ways, such as production costs, which not only depend on the availability of materials to produce the signal, but also on the internal

processes needed to organize these materials. These requirements can influence the expression of signals by extent of the production costs, evolving trade-offs between signal development and essential physiological functions, such as the immune defense or the antioxidant system. The energetic expenditure of producing and maintaining components of the immune system may have a serious burden on organization, thus creating a link between the immune system and condition-dependent sexual advertisement (Wedekind 1992; Wedekind and Folstad 1994). Relationships of sexual signals with immunity and the antioxidant system can be complex and highly dependent on the properties of materials that made the signal (e.g., pigments or pheromones).

With respect to visual signals, there are several hypotheses on the possible role of carotenoids as sexual signals, which are the most studied pigments in behavioral ecology. Since carotenoids are synthesized only by plants, algae, some bacteria and fungi (Goodwin 1986), but not by animals (Fox 1979; Moran and Jarvik 2010), carotenoids could be perfect signals of food availability and foraging ability. Also, several immunostimulatory properties of carotenoids, such as thymocytes activation (Garbe et al. 1992), the expression of immune-related genes (Geissmann et al. 2003), the upregulation of proteins involved in cell-to-cell communication (Basu et al. 2001) and the increase of membrane fluidity (Chew and Park 2004), support the existence of a trade-off between the carotenoid-based signal system and the immune defense. Moreover, since carotenoids have free radical scavenger properties in vitro (Krinsky and Yeum 2003), they might have a more complex role not only in immunity but also in the antioxidant functions of the body creating multiple trade-offs with sexual signals. However, the role of carotenoids as in vivo antioxidants is under debate, because carotenoids transform toxic aldehyds and lose their color by reaction with free radicals (Palozza 1998), while antioxidants without color (e.g., vitamin E or C) are more effective radical scavengers and vitamin E is supported by vitamin C to transform back to the original effective state (Buettner 1993; Bruno et al. 2005). Thus, carotenoids might have other more important roles in the body (e.g. as sexual signals, immunity, etc.) rather than being antioxidants. Nevertheless, carotenoids might be affected from free radicals if the

antioxidant system is not adequate. Therefore, carotenoid-based sexual signals might simply indicate visually the state of the true antioxidant system (Hartley and Kennedy 2004).

On the other hand, despite of melanins are the most common pigments in vertebrate tissues (Galván and Solano 2015), their role and exact function in immune and antioxidant defense are less known comparing to carotenoids, particularly in lower vertebrates (Mackintosh 2001; Slominski et al. 2004; Galván and Alonso-Alvarez 2009; Galván and Solano 2009). Melanins often reliably indicate social status (Santos et al. 2011; Diep and Westneat 2013), aggressiveness and sexual activity (Ducrest et al. 2008), which support the traditional view that melanin-based signals are badges that only elicit socially motivated costs (Maynard-Smith and Harper 2003). However, although melanins can be synthesized de novo in the body, melanin-based signals may also indicate foraging ability because availability of other compounds required for the production of melanins, such as phenylalanine, tyrosine, and calcium, may be limited (McGraw 2008). Melanins have important roles in tissue strengthening (Bonser 1995), photoprotection (in skin and eyes) (Ortonne 2002; Seagle et al. 2005; Reguera et al. 2014) and thermoregulation (Cloudsley-Thompson 1999; Reguera et al. 2014). Melanins, similarly to carotenoids, also have free radical scavenger properties and, furthermore, have important immunological roles in invertebrates and mammals (Mackintosh 2001; Slominski et al. 2004). Also, the expression of melanin-based signals can be influenced in several ways, such as being tightly controlled by genes, and melanin production is affected by hormones (α-melanocyte-stimulating hormone, testosterone) and intracellular antioxidants (glutathion) (Slominski et al. 2004; Galván and Alonso-Alvarez 2008; Galván and Alonso-Alvarez 2009; Galván and Solano 2009). Thereby, melanins may signal different aspects of the health state of the owner than carotenoid signals.

With respect to chemical signals, pheromone components also can be costly, particularly when important compounds are used for signaling, such as a precursor of vitamin D or α-tocopherol (=vitamin E) in lizards (Martín and López 2006; Kopena et al. 2011). Vitamin E has relevant roles in the immune defense of vertebrates, such as increasing antibody production, macrophag and complement activity, and limphocyte proliferation

(Surai 2002). Immune cells are rich in polyunsaturated fatty acids, which are very susceptible to the attacks of free radicals. Vitamin E increases membrane resistance against lysis activated by the innate immune system (Hong et al. 2004). Even the smallest lipid peroxidation can change membrane structure and its properties, inhibiting the antigen recognition, receptor expression, secretion of antibodies and cytokines, etc, thereby damaging the processes of the immune system (Wu and Meydani 1998). However, vitamin E is one the most useful fat-soluble antioxidants (Di Mascio et al. 1991), and, therefore, the presence of vitamin E in chemical signals may suffer important trade-offs that may render it as a honest signal (Kopena et al. 2011, 2014a,b).

There are several studies that have investigated the relationships of immunity and the antioxidant system with the sexual signals. However, unfortunately, results are controversial, particularly for carotenoid-based signals. Several studies have found a positive relationship between carotenoids and some aspects of the immune response in birds (Blount et al. 2003; McGraw and Ardia 2003; McGraw et al. 2006a) and fish (Amar et al. 2000; Clotfelter et al. 2007), but there are also many examples demonstrating a lack of association both in birds (Navara and Hill 2003; Biard et al. 2006; Hõrak et al. 2006; McGraw et al. 2006b; Sutherland et al. 2012), lizards (Kopena et al. 2014b) and fish (Lin et al. 2010). Unfortunately, there is very little information about relationships of melanin-based signals and the immune response, but these seem to be similar; melanin-based signals positively relate with the immune response in some birds and lizards (Jacquin et al. 2011; Vroonen et al. 2013). One of the possible reasons of these controversial results is that the body can react in different ways in a normal healthy situation than under an immune challenged situation. This is because the full antioxidant and immune capacity is only activated in case of an immune challenge (Ji 1999; Selman et al. 2000; Aguiló et al. 2005; Monaghan et al. 2009), which also increases oxidative stress by macrophags and neutrophils (Nathan and Shiloh 2000; Coleman 2001; Halliwell and Gutteridge 2015). Immune activity usually diverts carotenoids from blood and decreases carotenoid-based skin or beak coloration (Faivre et al. 2003; Alonso-Alvarez et al. 2004; Torres and Velando 2007; López et al. 2009a; Ibáñez et al. 2014). Furthermore, an immune challenge can also change composition of chemical

signals in lizards and mammals. For example, a vitamin D precursor found in chemical signals of male lizards *Podarcis hispanica*, which is preferred by females, decreases after an immune activation (López et al. 2009b). Immune activation changes volatile composition of urine in rodents, eliciting avoidance from conspecifics (Kimball et al. 2014; Boillat et al. 2015), and of sweat in humans in a way that females do not further prefer the modified pheromone (Olsson et al. 2014).

In this study, we examined in the Iberian or Schreiber's green lizard (*Lacerta schreiberi*) the effects of an immune challenge on visual and chemical sexual signals, and its relationship with the effects of the availability in the diet of a strong antioxidant, the vitamin E. This lizard is a large sexually dimorphic lacertid from the Iberian Peninsula. Males have, especially during the breeding season, green dorsal coloration with small black spots, yellow chest and ventral coloration, and bright blue and UV throat and mental coloration (Martín and López 2009), and produce femoral gland secretions with a high content of vitamin E (López and Martín 2006). In contrast, females are mainly dull brown, with pale yellow vents and do not have femoral secretion. Interindividual variation in the characteristics of males' breeding coloration and chemical signals may signal variation in morphology, parasite load, health state, dominance and pairing status (Martín and López 2009; Kopena et al. 2014a,b; Megía-Palma et al. 2016). In a previous study, we found that both carotenoids and vitamin E in the diet affect the expression of color ornaments and chemical signals of male *L. schreiberi* lizards (Kopena et al. 2014a). However, the addition of vitamin E alone has a higher effect on the expression of coloration of most of ornaments than the addition of carotenoids alone, even for those ornaments that are not carotenoid dependent but melanin-based (Kopena et al. 2014a). Also, vitamin E increases the PHA immune response, while carotenoids do not have this effect, although carotenoids improve body condition. This supports the hypothesis that carotenoids are not effective as immunostimulants, but that they may be just visually signaling the immunostimulatory effects of non-pigmentary antioxidants, such as the vitamin E (Kopena et al. 2014b).

Here, to look for immunity and antioxidant costs of sexual signals, we experimentally challenged the immune system of male lizards *L. schreiberi* with a bacterial

antigen (lipopolysaccharide; LPS), without pathogenic effects (Bonneaud et al. 2003; Alonso-Alvarez et al. 2004; Staszewski and Boulinier 2004; Loyau et al. 2005; Uller et al. 2006). We examined whether and how the characteristics of the lizards' visual and chemical sexual signals were affected by the activation of the immune and antioxidant systems. We predicted that if the visual and chemical sexual traits honestly reflected current health, and if there existed a trade-off between the immune and/or antioxidant functions and the elaboration of ornaments, the activation of the immune system should reduce the ''intensity'' of visual and chemical displays. Furthermore, we examined the interactive effects of a dietary supplementation of vitamin E in the immune challenged situation. We expected that the detrimental effects of the immune activation might be compensated by increasing the availability of vitamin E in the diet due to its immunostimulant and antioxidant roles.

Materials and methods

Study animals

In May 2013, we collected 60 adult male lizards *L. schreiberi* at two nearby valleys ('Valle de La Fuenfría' and 'Valle de Navalmedio') in the Guadarrama Mountains (40°44' N, 04°02' W; Madrid Province, Spain). We immediately transferred lizards to ''El Ventorrillo'' field station of the Museo Nacional de Ciencias Naturales (Madrid province, Spain), 5 km from the capture site. During all the study, lizards were individually kept in outdoor 51x36x28 cm PVC terraria containing coconut fiber substratum and rocks for cover. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*) and house crickets (*Acheta domesticus*), and water was provided ad libitum. The photoperiod and ambient temperature were those of the surrounding region. Before and at the end of the experiments, we measured male body weight with a digital balance to the nearest 0.1 g (initial, mean ± SE = 23.4 ± 5.6 g; range = 10.5-35.0 g) and used a digital caliper to measure snout-to-vent length (SVL) to the nearest 1 mm (initial, mean ± SE = 98 ± 8 mm; range = 76-111 mm). We calculated body condition before and after the experiment as the residuals of the lnBodyMass regression on lnSVL, and used changes in body condition through the experiment for posterior analyses.

Activation of the immune system and vitamin E supplementation

We designed this study to test the single and interactive effects of 1) the activation of the immune system induced by a lipopolysaccharide (LPS) injection and 2) the dietary supplementation of vitamin E. Thus, we had four experimental treatments: 'injection of LPS alone' (L), 'dietary supplementation of vitamin E alone' (E), 'supplementation of vitamin E and injection of LPS together' (EL), and 'control' (C). To control for differences in body size, we classified males in groups of four based on similar body length (difference of SVL among males within a quartet: mean = 0.6 mm, maximum = 1.3 mm). In every quartet, we selected randomly which individuals were assigned to each of the four treatments. All lizards were individually kept under similar captivity conditions.

To test for the effects of the activation of the immune system, in two treatments (L and EL), we used lipopolysaccharide (LPS) from the cell wall of the bacteria *Escherichia coli* (serotype 0111-B4; Sigma-Aldrich). LPS induces an inflammatory response by non-specifically activating B and T lymphocytes and producing specific anti-LPS antibodies but has no pathogenic effects (Janeway et al. 2001). However, LPS also induces immunopathological effects such as fever (Deen and Hutchison 2001). On the first day of the experiment, we injected intraperitoneally male lizards in the L and EL treatments with LPS (2.5 μg/g of body weight) diluted in 0.05 ml of phosphate buffered saline (PBS). The serotype used and the concentration were similar or lower to those previously used to stimulate the immune system in other lizards (Deen and Hutchison 2001; Uller et al. 2006; López et al. 2009a,b). On the same day, males in the other two treatments (E and C) were injected with the same volume (0.05 ml) of PBS alone as a control. Lizards were handled "blind" by the same investigator using the same protocol for injections.

To test for the effects of dietary vitamin E, lizards were supplemented in two of the treatments (E and EL) with vitamin E. During 18 days, lizards were fed daily 20 μL vitamin E supplement (synthetic (±)-α-tocopherol; purchased from Sigma-Aldrich Chemicals Co., St. Louis, Missouri, USA), which contained 97% of synthetic vitamin E (approx. 1014 IU mL^{-1}) and 3% soybean oil (with approx. 0.32 IU mL^{-1} of natural vitamin E, i.e., D-α-tocopherol). Thus, the daily dose provided for each male was approximately 20.2 IU of

vitamin E, which is close to the daily minimal physiological necessity of vitamin E for similar sized reptiles, and well below the tolerable upper intake levels (Mader 1996; Allen et al. 2004; Bender 2009; Kopena et al. 2014a,b). In the other two treatments (L and C), males were fed daily 20 µl soybean oil alone as a control. To ensure that all lizards ingested the same amount of vitamin or soybean solution, we gently handled lizards and used sterile plastic syringes with a canula to slowly deliver the solution into their mouth, thus ensuring that lizards swallowed the entire dose.

Color measurements

At the beginning and at the end of the experiments (18 days after the injection of LPS and after the first supplementation of vitamin E), we measured reflectance of lizards' coloration from 300 to 700 nm using an Ocean Optics USB2000 spectroradiometer with a DT-1000-MINI Deuterium–Halogen light source (Ocean Optics, Inc., Dunedin, FL, USA). This range of wavelengths includes the UV range and coincides with the spectral sensitivity of other lacertid lizard species (Pérez i de Lanuza et al. 2014, Martin et al. 2015). We used a cylindrical metallic tube mounted on the bifurcated fiber optic probe to exclude ambient light and standardize measuring distance and angle (Montgomerie et al. 2006). The probe was held at a 45° angle to the skin, and reflectance was always measured by the same person (PL). We measured coloration of three visually distinct large patches of coloration ("blue" throat, "yellow" chest, and "green" dorsum) at three standardized spots: the middle of the throat (between the last chin shields and the collar; "throat"), the chest (just anterior to the two forelimbs at the middle of the second row of scales prior to the collar; "chest"), and the dorsum (in the middle point above the two forelimbs; "dorsal") (Martín and López 2009; Kopena et al. 2014a). At each body location, we measured coloration of three nearby points and later calculated average values for throat, chest and dorsal coloration of each individual.

We used principal component analyses (PCAs) to summarize all of the information about the shape of the complex reflectance spectra, including bimodal ones like those of *L. schreiberi* (see Martín and López 2009), into three independent principal components (PCs) (Endler 1990; Cuthill et al. 1999; Grill and Rush 2000; Montgomerie 2006). In the PCA of

spectral data, PC1 represents variation in intensity of coloration or brightness, and the subsequent PCs represent combinations of hue and chroma (Endler 1990; Cuthill et al. 1999; Grill and Rush 2000). Moreover, the PCA identifies those sections of the spectrum (wavelength regions) that contribute to the observed variation, independently of their contribution to the total amount of reflectance (Montgomerie 2006). We considered separately each part of the body (throat, chest and dorsal) and, for each part, we made two different PCAs for colors measured before and after the experiment. Because initial values of coloration did not differ significantly among treatments in any case (ANOVAs for all PC scores for each of the three body parts, $P > 0.80$ for all), we only used in further analyses the results of the PCAs for color measured after the experimental treatment.

In addition, to clarify the role of carotenoids in changes of coloration in chest and dorsal coloration, we calculated the violet-blue reflectance of spectra ($R_{400–515}$) to measure carotenoid light absorbance (Shawkey et al. 2006, San-Jose et al. 2013) and the background reflectance of spectra ($R_{575-700}$) where carotenoids do not absorb light before and after the experiments. We used the ratio of violet-blue and background reflectance ($R_{violet-blue}/R_{background}$) as an indication of absolute carotenoid chroma (Jacot et al. 2010, San Jose et al. 2013). In case of an achromatically varying background, the absolute carotenoid chroma correlates negatively with saturation and reflects precisely the carotenoid content (Jacot et al. 2010). However, in case of colored backgrounds, since chromatic variation is not influenced only by carotenoids, the absolute carotenoid chroma may not predict reliably the carotenoid concentration of the skin (San-Jose et al. 2013). We subsequently correlated absolute carotenoid chroma values with the PC scores of chest and dorsal coloration to explore whether variation in coloration defined by these PC scores could be determined by carotenoid levels.

Chemical analyses of femoral secretions

Before and at the end (18 days after the first day) of the experimental treatments, we collected femoral secretion of males directly into glass vials with glass inserts, later closed with Teflon-lined stoppers and stored at -20°C. Samples were analyzed by gas

chromatography-mass spectrometry (Finnigan-ThermoQuest GC-MS Trace 2000, Thermo-Quest Corporation, Austin, Texas) equipped with a Supelco-Equity-5 (Bellefonte, PA) column temperature programmed (50 to 280 °C at 5 °C/min and 280 °C for 30 min). Compounds were identified by comparison of mass spectra in the NIST/EPA/NIH (NIST 02, www.nist.gov) library, and later confirmed with authentic standards (see López and Martín 2006 for details of analyses and chemical compounds in secretions of this lizard species).

The relative amount of α-tocopherol (= vitamin E) in femoral secretions was determined as the percent of the total ion current (TIC) area transformed following Aitchison's formula: $[Z_{ij}=\ln(Y_{ij}/g(Y_j)]$, where Z_{ij} is the standardized peak area i for individual j, Y_{ij} is the peak area i for individual j, and $g(Y_j)$ is the geometric mean of all peaks for individual j (Aitchison 1986) (for similar analyses see López et al. 2006).

Statistical analyses

We used one-way analyses of variance (ANOVAs) to test for differences among the four experimental treatments. Dependent variables were either body condition, the color PC scores of the three different body parts, violet-blue and background reflectance, absolute carotenoid chroma of chest and dorsal coloration or vitamin E content in femoral secretions. Pairwise comparisons used unequal N Tukey's honestly significant difference tests (Sokal and Rohlf 1995).

Results

Body condition

There were not initial significant differences in body condition of lizards among treatments (one-way ANOVA; $F_{3, 55} = 0.60$, $P = 0.61$). However, changes in body condition through the experiment among treatments were significant (one-way ANOVA; $F_{3, 54} = 4.12$, $P = 0.01$). Lizards supplemented with vitamin E and injected with LPS (EL) increased significantly less their body condition than control lizards (Tukey's tests, $P = 0.007$). The

rest of treatments showed intermediate increases in body condition, although comparisons did not reach significance ($P > 0.13$ for all) (Fig. 1).

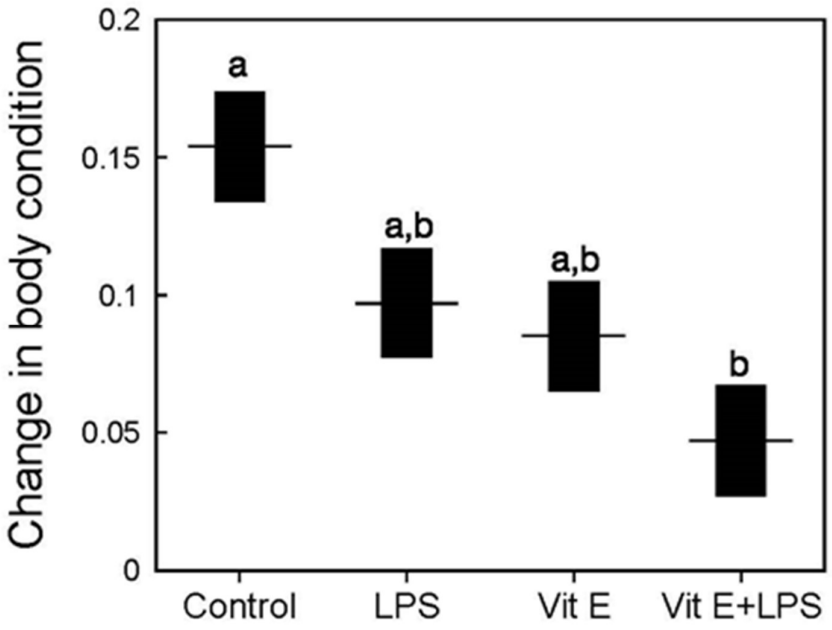

Fig. 1 Changes in body condition (mean ± SE) of male lizards *L. schreiberi* in each experimental treatment. Means with the same letter above the bars were not significant different.

Throat coloration

The PCA on reflectance data of all spectra of throat coloration at the end of the experiment produced three throat principal components (thPCs) that together accounted for 96.5 % of the variation in the original spectra. Coefficient relating to first PC (thPC1) (eigenvalue = 65.46; 80.8 % of the variation) to the original reflectance data were all negative and of similar magnitude, so thPC1 represented achromatic brightness variation in the original spectra (Fig. 2a). The coefficients related the second PC (thPC2) (eigenvalue = 7.67; 9.5 % of the variation) to the original reflectance values below 530 nm were all positives, while above 530 nm they were all negatives (Fig. 2a). The pattern of coefficients of the third PC (thPC3) (eigenvalue = 5.01; 6.2 % of the variation) represented variation in the relative amounts of medium (400-580 nm) wavelengths in the positive side to both very short (300-400 nm) and very long (580-700 nm) wavelengths in the negative side (Fig. 2a).

There were not significant differences among treatments in thPC1 (i.e., brightness) (one-way ANOVA; $F_{3, 55} = 0.61$, $P = 0.61$) (Fig. 3a). However, there were significant differences among treatments in thPC2 (one-way ANOVA; $F_{3, 55} = 7.36$, $P = 0.0003$) (Fig. 3b). Lizards in the two treatments that were supplemented with vitamin E (E and EL) did not differ between them (Tukey's test, $P = 0.58$) and had significantly higher values of thPC2 scores (i.e., higher amounts of short UV-blue wavelengths) than the control group ($P < 0.01$ for both), but they did not differ significantly from the L treatment ($P > 0.20$ for both). Lizards in the L treatment also tended, but not significantly, to differ from the control treatment ($P = 0.078$) (Fig. 3b). With respect to thPC3, there were also significant differences among treatments (one-way ANOVA; $F_{3, 55} = 3.81$, $P = 0.015$) (Fig. 3c). Lizards injected with LPS (L and EL treatments) had significantly lower values of thPC3 scores (i.e., higher amount of very short UV wavelengths) than the control group (Tukey's tests, $P < 0.04$ for both), but the rest of comparisons were not significant ($P > 0.38$ for all) (Fig. 3c).

Chest coloration

The PCA on reflectance data of all spectra of chest coloration at the end of the experimental treatments produced three chest principal components (chPCs) that together accounted for 95.1 % of the variation in the original spectra. The first PC (chPC1) (eigenvalue = 54.92; 67.80 % of variation) had negative coefficients relating chPC-1 to the original reflectance data, thus representing achromatic brightness (Fig. 2b). The coefficients relating the second PC (chPC2) (eigenvalue = 19.34; 23.88 % of the variation) to the original reflectance values below 510 nm were all positive, while above 510 nm they were negative (Fig. 2b). Thus, chPC2 represented variation in the relative amount of short- to long-wavelength reflectance, with lower chPC2 scores indicating more saturated "yellowish" colors. Finally, the pattern of coefficients of the third PC (chPC3) (eigenvalue = 2.74; 3.38% of the variation) represented variation in the relative amounts of both short (350-515 nm) and very long (610-700 nm) wavelengths in the negative side to both very short (300-350)

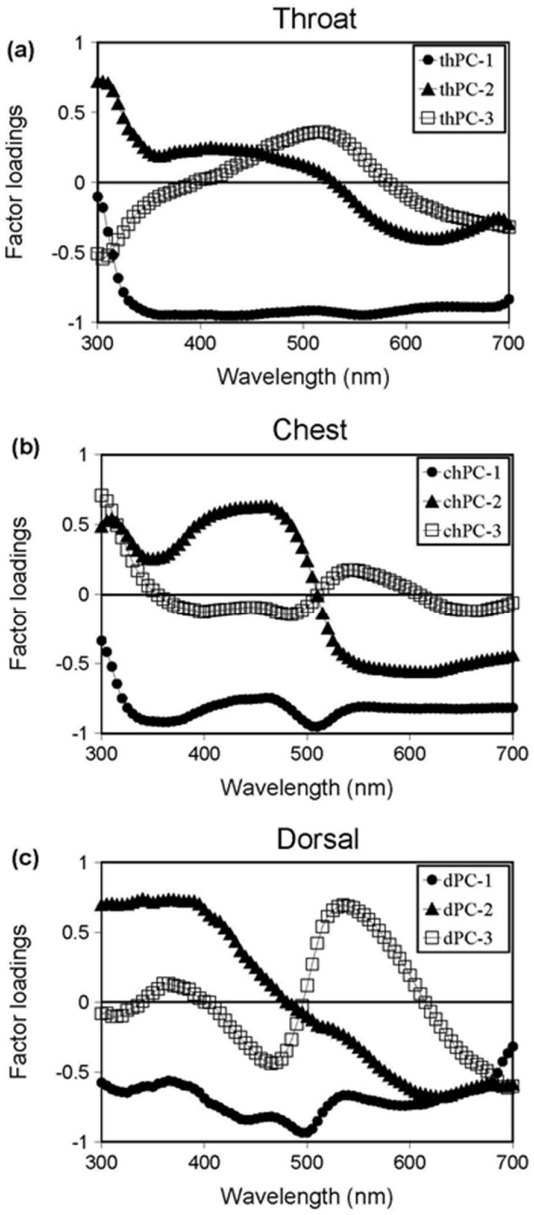

Fig. 2 Coefficients of the first three principal components from PCAs on reflectance spectra that characterize throat (**a**), chest (**b**), and dorsal (**c**) coloration of male lizards *L. schreiberi.*

and medium (515-610 nm) wavelengths in the positive side (Fig. 2b). Absolute carotenoid chroma was significantly highly and negatively correlated with chPC1 (r = -0.46, P= 0.0002) and significantly highly and positively correlated with chPC2 (r = 0.84, P < 0.0001).

There were significant differences among treatments in chPC1 (i.e., brightness) (one-way ANOVA; $F_{3, 55}$ = 3.79, P = 0.015), with lizards supplemented with vitamin E alone (E) having significantly brighter chests than the control group and that lizards injected with LPS alone (L) (Tukey's test, P < 0.04 for both), while the other comparisons were not

significant ($P > 0.13$ for all) (Fig. 4a). Also, there were significant differences among treatments in chPC2 (one-way ANOVA; $F_{3, 55} = 5.06$, $P = 0.0036$); lizards supplemented with vitamin E and injected with LPS (EL) had significantly lower amounts of long "yellowish" wavelengths in the chest than the control group (Tukey's test, $P = 0.002$) and

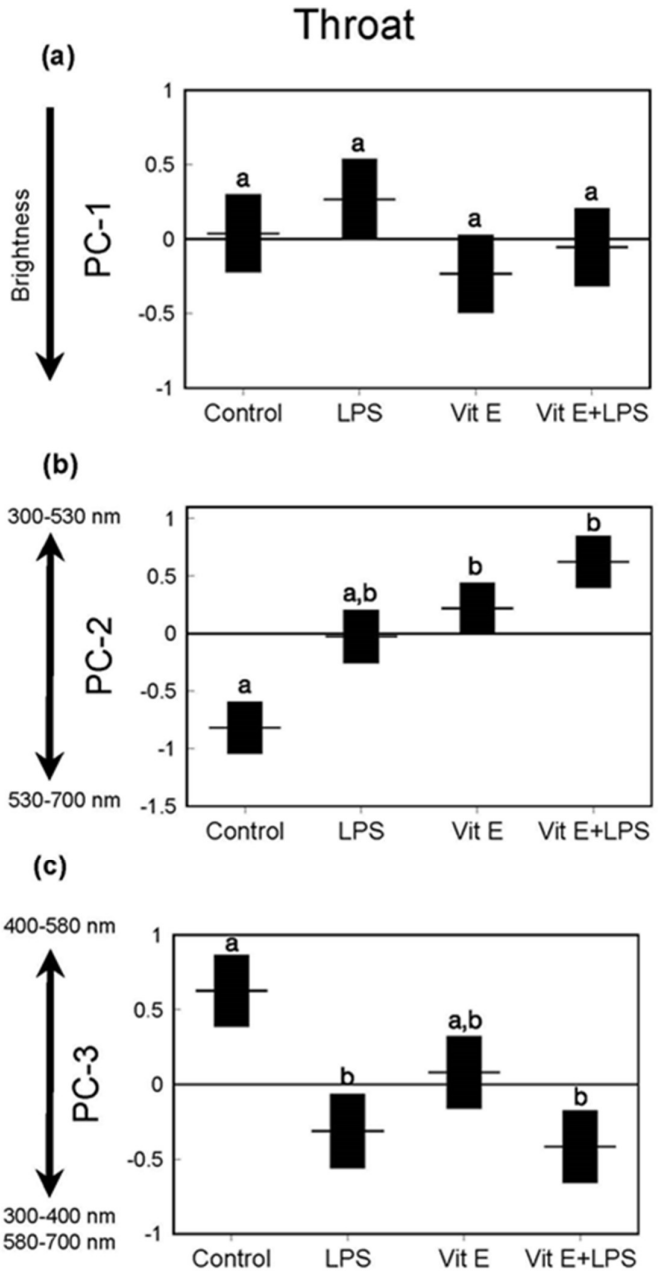

Fig. 3 Mean (±SE) of thPC scores describing characteristics of throat coloration of male lizards *L. schreiberi* at the end of each experimental treatment. Arrows indicate the variables or wavelengths correlated with each PC. Means with the same letter above the bars were not significant different.

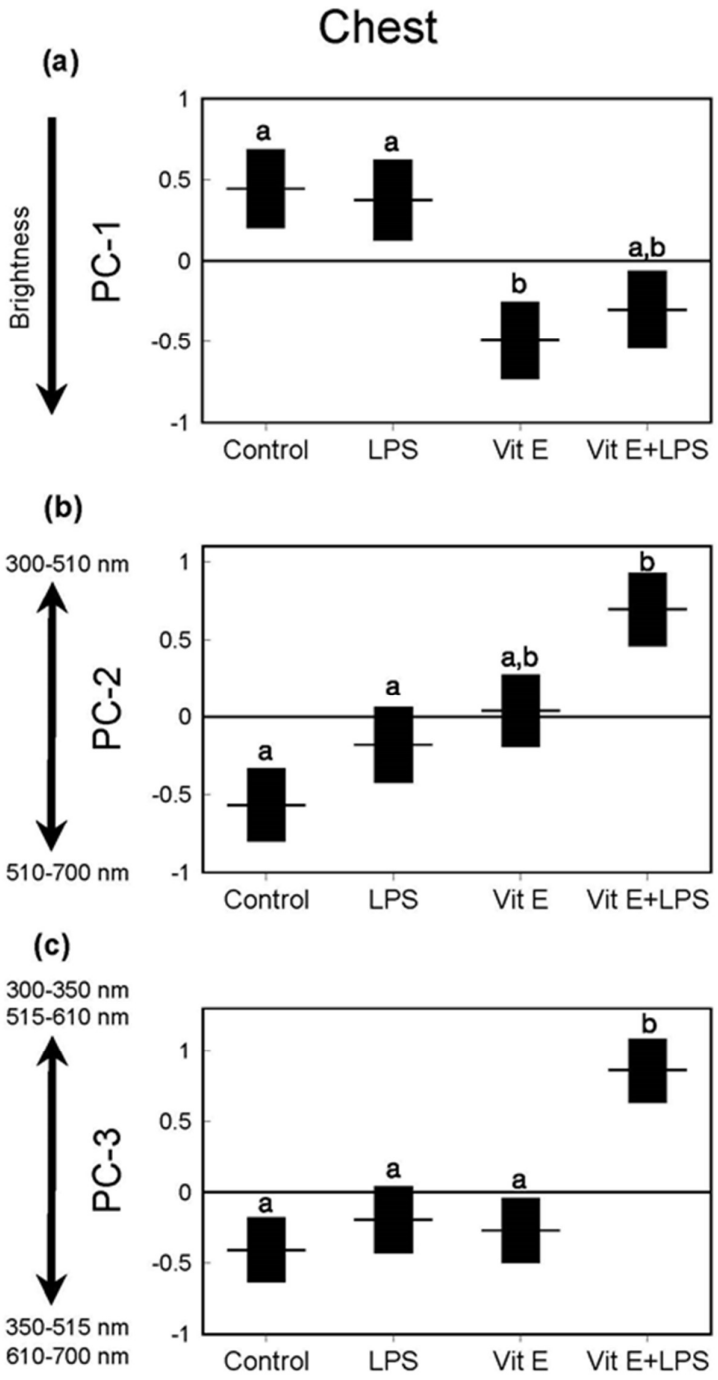

Fig. 4 Mean(±SE) of chPC scores describing characteristics of chest coloration of male lizards *L. schreiberi* at the end of each experimental treatment. Arrows indicate the variables or wavelengths correlated with each PC. Means with the same letter above the bars were not significant different.

that lizards injected with LPS alone (L) ($P < 0.05$), but the rest of comparisons were no significant ($P > 0.21$) (Fig. 4b). Also, there were significant differences in chPC3 among treatments (one-way ANOVA; $F_{3, 55} = 6.50$, $P = 0.0008$), with lizards in the EL treatment

having significantly higher values of chPC3 scores (i.e., higher amount of medium wavelengths) than the other groups (Tukey's tests, $P < 0.01$ for all) while the other treatments did not differ among them ($P > 0.91$ for all) (Fig. 4c).

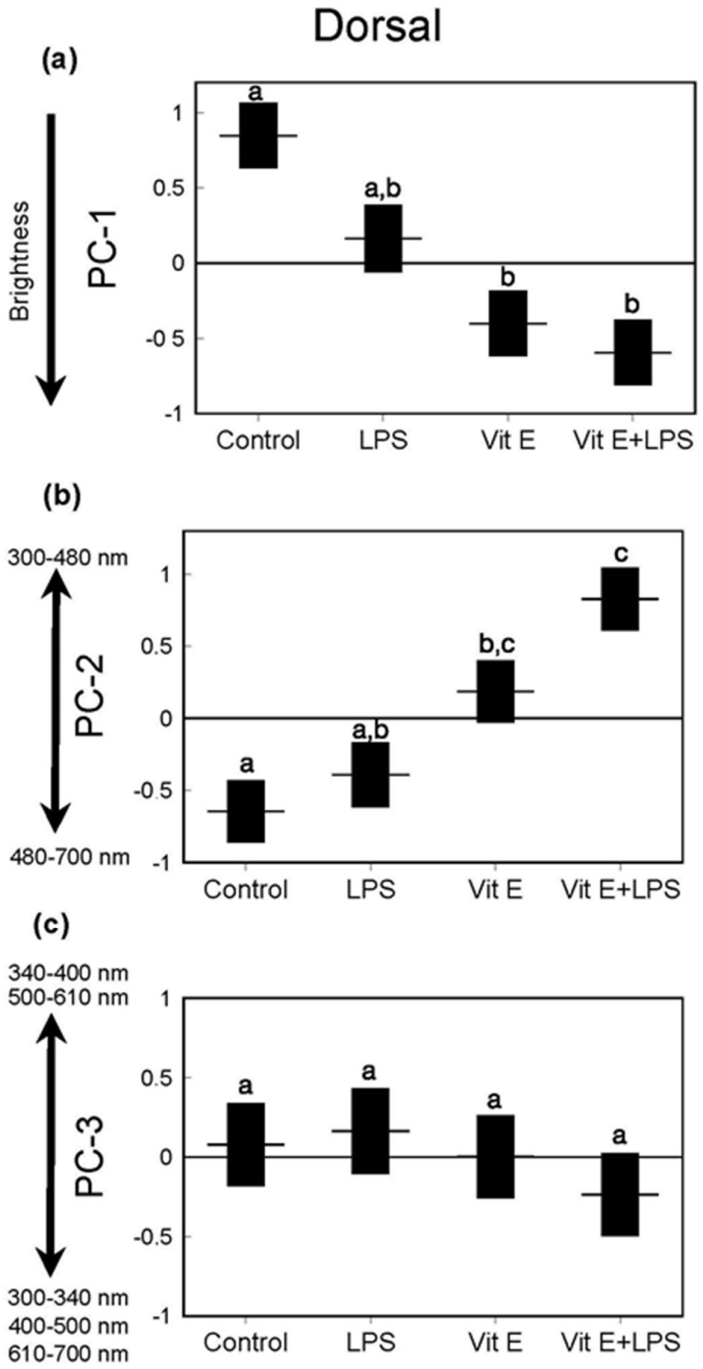

Fig. 5 Mean(±SE) of dPC scores describing characteristics of dorsal coloration of male lizards *L. schreiberi* at the end of each experimental treatment. Arrows indicate the variables or wavelengths correlated with each PC. Means with the same letter above the bars were not significant different.

We found significant differences among treatments in temporal changes in violet-blue reflectance (one-way ANOVA; $F_{3, 55} = 8.62$, $P < 0.001$); lizards supplemented with vitamin E and injected with LPS (EL) decreased significantly less the violet-blue reflectance in the chest than the control group (Tukey's test, $P < 0.001$) and that lizards injected with LPS alone (L) ($P = 0.003$), and tended to decrease it although non-significantly less than the group supplemented with vitamin E ($P = 0.053$), but the rest of comparisons were no significant ($P > 0.14$). There were no significant differences among treatments in temporal changes of background reflectance (one-way ANOVA; $F_{3, 55} = 1.18$, $P = 0.327$). Changes in absolute carotenoid chroma showed significant differences among treatments (one-way ANOVA; $F_{3, 55} = 10.94$, $P < 0.001$) (Fig. 6a), the control group differed significantly in change of absolute carotenoid chroma from the group supplemented with vitamin E alone (E) (Tukey's test, $P = 0.012$) and from the group supplemented with vitamin E and injected with LPS (EL) ($P < 0.001$). Furthermore, this latter group (EL) differed significantly in absolute carotenoid chroma from the group with immune challenge alone (L) ($P < 0.001$), but the rest of comparisons were no significant ($P > 0.11$).

Dorsal coloration

The PCA on reflectance data of all spectra of dorsal coloration at the end of the experiment produced three dorsal principal components (dPCs) that together accounted for 92.56 % of the variation in the original spectra. The first PC (dPC1) (eigenvalue = 40.39; 49.87 % of variation) represented achromatic brightness variation (Fig. 2c). The pattern of coefficients of the second PC (dPC2) (eigenvalue = 23.99; 29.63 % of the variation) suggested it represented variation in the relative amounts of short wavelengths (300-480 nm) in the positive side to long wavelengths (480-700 nm) in the negative side (Fig. 2c). Absolute carotenoid chroma was significantly highly and negatively correlated with dPC1 ($r = -0.43$, $P = 0.0008$), but significantly highly and positively correlated with dPC2 ($r = 0.75$, $P < 0.0001$). Thus, dPC2 represented variation in the relative amount of short- to long-wavelength reflectance, with lower dPC2 scores indicating more saturated "greenish" coloration, which probably depended on carotenoids. The third PC (dPC3) (eigenvalue =

10.58; 13.06 % of the variation) represented variation in the relative amounts of both very short (300-340 nm), medium (400-500 nm) and very long (610-700 nm) wavelengths in the negative side to short (340-400 nm) and long (500-610 nm) wavelengths in the positive side, with lower dPC3 scores indicating more saturated "greenish" colors (Fig. 2c).

There were significant differences among treatments in dPC1 (one-way ANOVA; $F_{3, 55} = 8.85$, $P < 0.0001$), with lizards supplemented with vitamin E (E and EL) having significant brighter dorsal coloration than the control group (Tukey's tests, $P = 0.001$ for both), but the rest of comparisons were not significant ($P > 0.08$ for all) (Fig. 5a). Similarly,

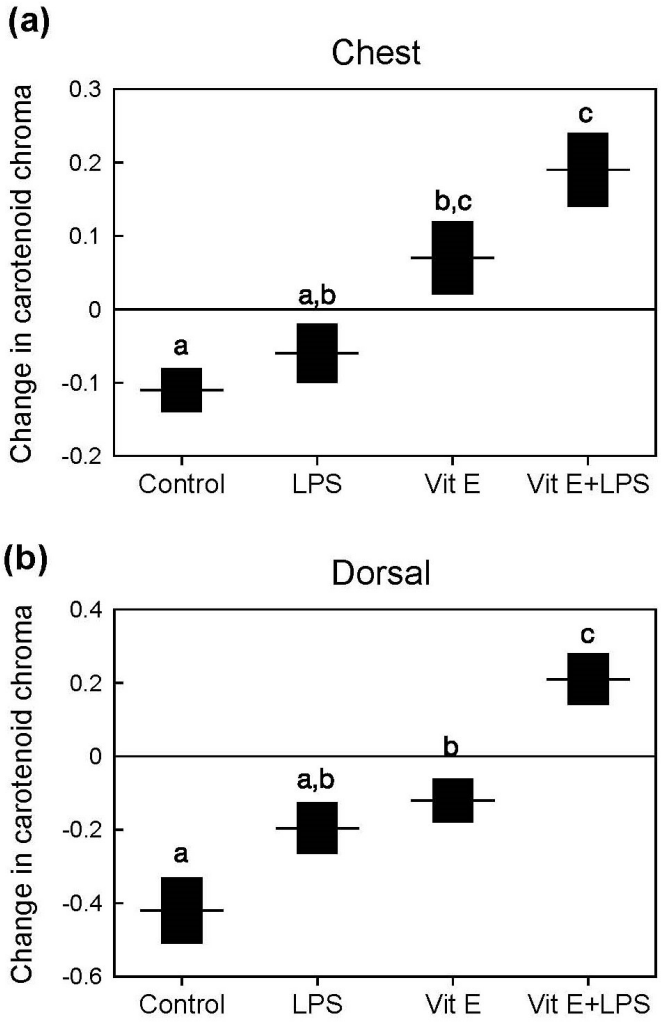

Fig. 6 Mean(±SE) of change in absolute carotenoid chroma on chest (**a**) and dorsal coloration (**b**) of male lizards *L. schreiberi* in case of each experimental treatment. Means with the same letter above the bars were not significant different.

with respect to dPC2, there were significant differences among treatments (one-way ANOVA; $F_{3, 55} = 9.05$, $P < 0.0001$) (Fig. 5b). Lizards supplemented with vitamin E (E and EL) had significantly higher amounts of short wavelengths in dorsal coloration (i.e., they were "less greenish") than the control group (Tukey's tests, $P = 0.04$ and $P = 0.0002$ respectively), lizards injected with LPS but supplemented with vitamin E (EL) had significantly higher amounts of short wavelengths than lizards injected with LPS alone (L) ($P = 0.0016$), and the rest of comparisons were no significant ($P > 0.16$ for all) (Fig. 5b). Finally, there were not significant differences among treatments in dPC3 (one-way ANOVA; $F_{3, 55} = 0.42$, $P = 0.74$) (Fig. 5c).

We found significant differences among treatments in the temporal change of violet-blue reflectance (one-way ANOVA; $F_{3, 55} = 3.56$, $P = 0.02$); lizards supplemented with vitamin E and injected with LPS (EL) decreased significantly less the dorsal violet-blue reflectance than the control group (Tukey's test, $P = 0.018$) but the rest of comparisons were no significant ($P > 0.10$). There were no significant differences among treatments in change of background reflectance (one-way ANOVA; $F_{3, 55} = 0.57$, $P = 0.63$). There were significant differences in change of absolute carotenoid chroma among treatments (one-way ANOVA; $F_{3, 55} = 12.35$, $P < 0.001$) (Fig. 6b). The group supplemented with vitamin E alone (E) decreased significantly less the absolute carotenoid chroma than the control group (Tukey's test, $P = 0.03$). Furthermore, the group supplemented with vitamin E and injected with LPS (EL) differed significantly from the group supplemented with vitamin E alone ($P = 0.014$), from the control group ($P < 0.001$) and from the group with immune challenge alone (L) ($P = 0.002$), but the rest of comparisons were no significant ($P > 0.11$). Furthermore, we found a highly significant positive correlation between the change of absolute carotenoid chroma in chest and dorsal coloration ($r = 0.75$, $P < 0.0001$).

Vitamin E in femoral secretions

Initial relative proportions of α-tocopherol (= vitamin E) in femoral secretions of lizards did not differ significantly among treatments (one-way ANOVA; $F_{3, 56} = 0.20$, $P = 0.89$). However, at the end of the experiment, there were significant differences in

proportions of α-tocopherol among treatment (one-way ANOVA; $F_{3, 56} = 17.12$, $P < 0.0001$) (Fig. 7). There were two clear groups of lizards; the two treatments that received supplementary vitamin E (E and EL) had significantly higher proportions of α-tocopherol in their femoral secretions than the other two treatments (C and L) (Tukey's tests, $P < 0.0015$ for all). The rest of comparisons were not significant ($P > 0.30$ for all).

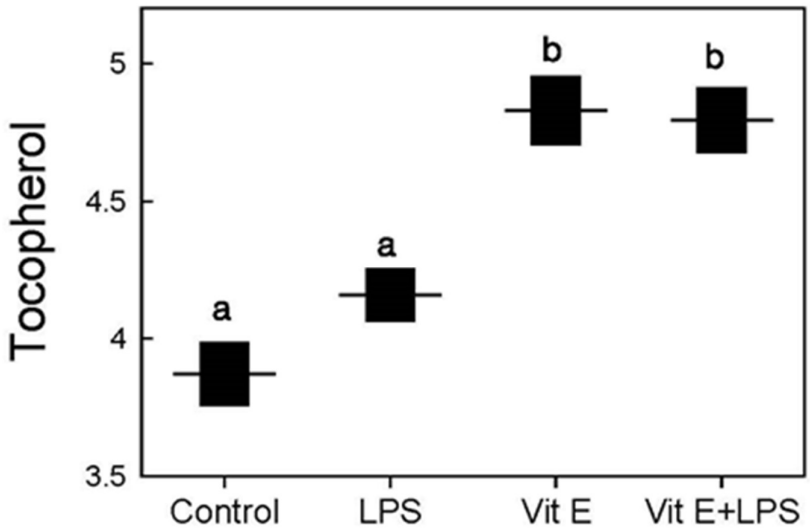

Fig. 7 Relative proportions (mean±SE of transformed TIC area) of α-tocopherol in femoral secretions of male lizards *L. schreiberi* at the end of each experimental treatment. Means with the same letter above the bars were not significant different.

Discussion

The observed effects of the immune challenge and the vitamin E dietary supplementation on sexual signals were surprising and in many aspects contrary to predictions. On the one hand, lizards that suffered an immune challenge did not decrease intensity of all sexual signals but, contrary to expected, increased saturation of throat coloration, particularly when challenged lizards had also been supplemented with vitamin E. Furthermore, vitamin E improved other characteristics of visual signals than those observed in previous experiments of dietary supplementation when climatic conditions in the year of study were very different (Kopena et al. 2014a). Also, some of the effects of vitamin E

depended on whether lizards were under an immune challenged situation, but others were entirely independent of this.

Based on studies of a closely related species of green lizard (*L. viridis*), it seems that the males' throat UV-blue coloration is the most preferred visual signal by females during mate choice (Bajer et al. 2010, 2012; Molnár et al. 2012), although other possible signals have been less studied (Martín and López 2009; Molnár et al. 2013). This UV-blue results from a structural coloration that depends on the nanostructure of a layer of iridophores and on the melanin content of a layer of melanophores below them (Bagnara et al. 1968; Quinn and Hews 2003; Megía-Palma 2016). In the current experiment, we found that vitamin E increased saturation of UV-blue coloration but not brightness, although an earlier study found that vitamin E increased brightness, but not the UV-blue saturation (Kopena et al. 2014a). However, in the current study, it could be possible that the increase of melanin elicited a decrease in the brightness that hid the positive effect of vitamin E for brightness. A possible reason for changes in throat coloration can be nanostructural changes in platelet distances and spacing. However, an increase in melanin-content may be more likely, because the changes in coloration observed here agree with the changes found in a study where melanin-content of *L. schreiberi* skin was decreased *in vitro* by oxidation (Megía-Palma 2016). In that study, it was found that melanin extraction from throat blue skin increased brightness and decreased short wavelength of the spectrum relative to long wavelength. In the current study, we observed the opposite process; brightness decreasing and short wavelengths increasing in the spectrum, possibly because of melanin increasing, although this was not supported by a previous study. Melanin production occurs in several different ways. One of them is by decreasing glutathione level, which is the most important intracellular antioxidant (Anderson 1998; Wu et al. 2004). Thereby, when glutathione level is high, the melanin production is inhibited, but when glutathione level is low, melanin production is started in melanocytes. But, with decreasing glutathione level, the body requires another effective antioxidant (e.g. vitamin E in our study) to substitute the antioxidant effects of glutathione in the cells. Otherwise, if there is a lack of an alternative antioxidant, the body would suffer a very high oxidative stress (Halprin and Ohkawara 1966;

Benedetto et al. 1981; Galván and Alonso-Alvarez 2008). The immune challenge alone also increased throat UV-blue saturation, which was also likely due to increases in melanin. This could be explained because histamine, which is released as the first reaction of the body in case of an immune activation, and nitrogen-oxide, which is released by macrophages and neutrophils after the immune activation by LPS (Tsatmali et al. 2000), have positive effects on melanin production, at least in mammals (Sasaki et al. 2000; Yoshida et al. 2000). Free radicals also can decrease glutathione, resulting in melanin increasing in melanophores. However, without substituting antioxidants, the body would suffer heavy oxidative stress with heavy costs for lifetime. Thus, cheating in melanin based signals can be very costly. When there is a lack of other alternative antioxidants, it is worth to maintain high glutathione levels, resulting in less intensive melanin-based coloration (Galván and Alonso-Alvarez 2008). Other possible way for this surprising reaction to the immune challenge is a genetical link between melanin production and immunity. Several diseases cause both albinism and impaired immunity (Introne et al. 1999; Baumeister et al. 2000; Spritz 2000). This can be possible by a linkage disequilibrum between melanogenic genes and immune function regulator genes or by pleiotropical genes that can simultaneously affect melanin-based coloration and immunity (Roulin 2015). However, throat PC3 coloration was also affected by the immune challenge, which increased very short UV wavelengths of the throat coloration. This may be explained by nanostructural changes in the iridophore layer, because neural and hormonal changes can affect distance of platelets, at least in chameleons (Teyssier et al. 2015). Furthermore, environmental stressors can affect development of structural coloration (Kemp and Rutowski 2007; Mäthger and Hanlon 2007). The effects of the immune challenge and the vitamin E supplementation were additive; the group with both manipulations (EL) had the “highest” saturation of UV-blue coloration.

The immune challenge alone did not affect chest coloration, but when it was combined with a supplement of vitamin E, it had a strong effect, decreasing yellowish saturation, carotenoid absorbance and increasing absolute carotenoid chroma. The immune challenge likely increased free radical levels and carotenoids were bleached. Thus, the supplement of vitamin E was not useful or was not used for defending carotenoids from

oxidation. The effects of vitamin E alone on chest coloration also differed from a previous study (Kopena et al. 2014a), with brightness increasing in the current, but not in the former, experiment. Furthermore, vitamin E did not change PC2 chest coloration and decreased carotenoid-content of the skin in the current year, while in a previous study made two years before there was a positive effect on chest PC2 and carotenoid chroma increased (Kopena et al. 2014a).

Changes in dorsal coloration were similar to alterations in chest coloration but more defined. Despite of in a previous study, vitamin E had no effect on dorsal coloration (Kopena et al. 2014a), in the current study we found that vitamin E alone increased brightness but decreased green saturation and carotenoid absorbance,which reveals a reduction of carotenoid content of the skin. The immune challenge alone did not have effects on dorsal coloration, but when it was combined with vitamin E supplementation, brightness increased and green saturation and carotenoid absorbance decreased, and absolute carotenoid chroma increased, likely because carotenoid content of xanthophores decreased. Although we do not know whether chromatic variations of dorsal background reflectance can bias change of absolute carotenoid chroma, the strong positive correlation with change of absolute carotenoid chroma in chest coloration with likely achromatic varying background may confirm the carotenoid base of the observed color change.

Body condition showed closely significant patterns, with vitamin E supplemented groups having lower values of body condition. Vitamin E needs lipids to be absorbed and transported in the body, and, furthermore, the experimental groups with vitamin E showed relevant alterations of coloration, which processes also need energy. Thus, supplemented males may need to use more of the energy acquired from food during the experiment. In contrast, it seems that the immune challenge did not affect fat stores. Nonetheless several earlier studies found in other animals that the immune activation by LPS elicited anorexia, higher glucose metabolism, and decreased activity (Feingold and Grunfeld 1992; Huang et al. 1999; Dantzer 2001). However, in our laboratory environment, where animals were fed and supplemented with soybean oil as a control, this fat store decreasing effect of immune

challenge could disappear due to the availability of more food than would be consumed in a natural habitat under a sickness condition (French et al. 2007; Ruiz et al. 2011).

With respect to chemical signals, vitamin E content in femoral secretions increased in the two vitamin E treatment groups, which is congruent with previous findings (Kopena et al. 2014a). This result supports the hypothesis that an increase in dietary nonpigmentary antioxidants will be directly reflected in the femoral secretions. Because female green lizards seem to be attracted to areas scent-marked by males with more vitamin E in secretions, it was suggested that this compound may act as an honest signal of male quality (Kopena et al. 2011). Vitamin E in secretions may signal the ability to obtain sufficient antioxidants, such that only males of high quality could allocate large amounts of vitamin E to the femoral secretions without causing a concomitant reduction in their antioxidant capacity (Brigelius-Flohe and Traber 1999; Martín and López 2010; Kopena et al. 2011). Interestingly, the increase of vitamin E in secretions occurred independently of that lizards had or had not been immune challenged. However, in other lizards and in rodents, an immune challenge affected negatively to the compounds of chemical signals that render scent attractive to females (López et al. 2009b; Kimball et al. 2014; Boillat et al. 2015). This could suggest that, in our study, the amount of the dietary supplementation was enough large as to compensate any negative effects.

Together interpreting the observed changes of sexual signals, we can see that dietary vitamin E provided an increase in melanin-based coloration in throat against a decrease in carotenoid-pigment coloration of chest and back. However, the increasing in brightness and in the UV range of chest and dorsal coloration can have positive effects on conspicuousness of male signals for females. Therefore, supplemented lizards may allocate energy to throat coloration, which may be the most important visual signal for *L. schreiberi*, as it occurs in related green lizards (Bajer et al. 2010, 2012; Molnár et al. 2012). This may be confirmed by the iridescence nature of throat coloration (Pérez i de Lanuza and Font 2014) requiring more precise nanostructure organization and increasing production and maintenance cost (Doucet and Meadows 2009). Nonetheless, although the death of one challenged male suggested that the immune activation was a relevant challenge for the body, challenged

males also increased intensity of sexual signals. Males with an immune challenge might try to maximize current fitness by increasing signal intensity in a situation where long-term expectatives of survival and future reproduction are low. A similar surprising result was found in the common lizard (*Lacerta vivipara*), where red ventral coloration signaling male quality increased by corticosterone, which activates physiological stress response and showed positive correlation with lipid peroxidation levels (Cote et al. 2010). However, why the immune challenge alone did not affect carotenoid-based signals is still unsolved. In goldfinches (*Carduelis tristis*) (Navara and Hill 2003) and in great tits (*Parus major*) (Fitze et al. 2007), it was also found a lack of effects of an immune challenge on feather carotenoid accumulation. However, this might be explained because feathers are not able to change rapidly the carotenoid content (Toomey et al. 2010). In our previous study, dietary supplementation of carotenoids had positive effects on condition but had no effects on PHA induced immune response of green lizards (Kopena et al. 2014b). So, in our laboratory experiment, males might have obtained enough energy from provided food to maintain the positive correlation between condition and carotenoid-dependent visual signals. Thus, this carotenoid signal may be condition-dependent rather than immunity-dependent. In the case of the vitamin E supplemented groups, this energy might be used for vitamin E transport and signal development, which would decrease condition implying a decrease of the carotenoid content. The most relevant alterations of coloration were observed in the immune challenged group that also received supplementary vitamin E. We found similar effects than in the vitamin E alone group, but more intense. This can be explained by the same effects than in the immune challenged alone group, but, in the combined treatment, males would have an additional source of vitamin E to increase femoral secretion and to act as an effective antioxidant to substitute glutathione in visual signals. In contrast, the price of a higher throat color saturation seems to be the carotenoid decreasing in other parts of the body that, nevertheless, seems to be less important as sexual signals in these lizards. However, the increasing of brightness and the UV of other visual signals can help females to perceive males by contrast with the environment more easily and this can help to increase mating possibilities.

We found conspicuous differences when comparing vitamin E effects in this study with an earlier experiment made on the same population (Kopena et al. 2014a). One of the possible reasons is the important differences in weather conditions between the two years. The current study was made in a very cold and wet spring ($T_{average\text{-}May2013}$: 9.3ºC), while the previous study was made in an "average" spring ($T_{average\text{-}May2011}$: 13.3ºC, while $T_{average\text{-}May2009\text{-}2015}$: 12.5ºC). The different temperature and sunbathing possibilities may change the structural development and melanin production of the skin. Furthermore, due to the different environmental circumstances, it is possible that females might prefer different male signals according to the differences in availability of food or raw elements of signals. In a cold, unsunny year, structural throat coloration can be a more important signal because UV saturation is highly affected by temperature (Bajer et al. 2012) and likely sunbathing. Furthermore, suboptimal body temperatures can increase free radical levels (Ballen et al. 2012). This signal can indicate a good quality home range with good sunbathing possibilities and less developmental stress (Kemp and Rutowski 2007; Mäthger and Hanlon 2007). Flexible mate choice by females is likely not a rare phenomenon, however less investigated (Kahn et al. 2013). In lark buntings (*Calamospiza melanocorys*), females shift preferences on male traits across years to increase their reproductive success (Chaine and Lyon 2008). In collared flycatchers (*Ficedula hypoleuca*), the development of different sexual badges was influenced by fluctuating climate conditions in different climatic areas (Garant et al. 2004; Hegyi et al. 2007). Flexible mate choice in *L. schreiberi* might allow females to choose the male trait that would maximize her fitness under the environmental conditions of each particular year. This adaptive plasticity in female mate choice might explain the evolution and maintenance of multiple types of sexual signals in this species.

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Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Captures and experiments were performed under license (Ref. 10/016732.9/13) from the Environmental Agency of Madrid Government ("Consejería del Medio Ambiente y Ordenación del Territorio de la Comunidad de Madrid", Spain).

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Conflicts of interest

Authors RK, PL and JM declare that they have no actual or perceived conflicts of interest.

References

Aguiló A, Tauler P, Fuentespina E, Tur JA, Córdova A, Pons A (2005) Antioxidant response to oxidative stress induced by exhaustive exercise. Physiol Behav 84:1–7

Aitchison J (1986) The statistical analysis of compositional data. Chapman and Hall, London

Allen DG, Pringle JK, Smith DA, Pasloske K, Day K (1998) Handbook of veterinary drugs. Lippincott-Raven Publishers, Philadelphia

Alonso-Alvarez C, Bertrand S, Devevey G, Gaillard M, Prost J, Faivre B, Sorci G (2004) An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. Am Nat 164:651–659

Amar EC, Kiron V, Satoh S, Okamoto N, Watanabe T (2000) Effects of dietary β-carotene on the immune response of rainbow trout *Oncorhynchus mykiss*. Fish Sci 66:1068–1075

Anderson ME (1998) Glutathione: an overview of biosynthesis and modulation. Chem Biol Interact 111:1–14

Bagnara JT, Taylor JD, Hadley ME (1968) The dermal chromatophore unit. J Cell Biol 38:67–79

Bajer K, Molnár O, Török J, Herczeg G (2010) Female European green lizards (*Lacerta viridis*) prefer males with high ultraviolet throat reflectance. Behav Ecol Sociobiol 64:2007–2014

Bajer K, Molnár O, Török J, Herczeg G (2012) Temperature, but not available energy, affects the expression of a sexually selected ultraviolet (UV) colour trait in male European green lizards. PloS One 7:e34359

Ballen C, Healey M, Wilson M, Tobler M, Wapstra E, Olsson M (2012) Net superoxide levels: steeper increase with activity in cooler female and hotter male lizards. J Exp Biol 215:731–735

Basu HN, Del Vecchio AJ, Flider F, Orthoeter FT (2001) Nutritional and potential disease prevention properties of carotenoids. J Am Oil Chem Soc 78:665–675

Baumeister FAM, Stachel D, Schuster F, Schmid I, Schaller M, Wolff H, Weiss M, Belohradsky BH (2000) Accelerated phase in partial albinism with immunodeficiency (Griscelli syndrome): genetics and stem cell transplantation in a 2-month-old girl. Eur J Pediatr 159:74–78

Bender DA (2003) Nutritional biochemistry of the vitamins. Cambridge University Press, Cambridge

Benedetto JP, Ortonne JP, Voulot C, Khatchadourian C, Prota G, Thivolet J (1981) Role of thiol compounds in mammalian melanin pigmentation: Part I. Reduced and oxidized glutathione. J Invest Dermatol 77:402–405

Biard C, Surai PF, Møller AP (2006) Carotenoid availability in diet and phenotype of blue and great tit nestlings. J Exp Biol 209:1004–1015

Blount JD, Metcalfe NB, Birkhead TR, Surai PF (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300:125–127

Boillat M, Challet L, Rossier D, Kan C, Carleton A, Rodriguez I (2015) The vomeronasal system mediates sick conspecific avoidance. Curr Biol 25:251–255

Bonneaud C, Mazuc J, Gonzalez G, Haussy C, Chastel O, Faivre B, Sorci G (2003) Assessing the cost of mounting an immune response. Am Nat 161:367–379

Bonser RH (1995) Melanin and the abrasion resistance of feathers. Condor 97:590–590

Brigelius-Flohe R, Traber MG (1999) Vitamin E: function and metabolism. Faseb J 13:1145–1155

Bruno RS, Ramakrishnan R, Montine TJ, Bray TM, Traber MG (2005) α-tocopherol disappearance is faster in cigarette smokers and is inversely related to their ascorbic acid status. Am J Clin Nutr 81:95–103

Buettner GR (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol, and ascorbate. Arch Biochem Biophys 300:535–543

Chaine AS, Lyon BE (2008) Adaptive plasticity in female mate choice dampens sexual selection on male ornaments in the lark bunting. Science 319:459–462

Chew BP, Park JS (2004) Carotenoid action on the immune response. J Nutr 134:257–261

Clotfelter ED, Ardia DR, McGraw KJ (2007) Red fish, blue fish: trade-offs between pigmentation and immunity in *Betta splendens*. Behav Ecol 18:1139–1145

Cloudsley-Thompson JL (1999) Multiple factors in the evolution of animal coloration. Naturwissenschaften 86:123–132

Coleman JW (2001) Nitric oxide in immunity and inflammation. Int Immunopharmacol 1:1397–1406

Cote J, Meylan S, Clobert J, Voituron Y (2010) Carotenoid-based coloration, oxidative stress and corticosterone in common lizards. J Exp Biol 213:2116–2124

Cuthill IC, Bennett ATD, Partridge JC, Maier EJ (1999) Plumage reflectance and the objective assessment of avian sexual dichromatism. Am Nat 153:183–200

Dantzer R (2001) Cytokine-induced sickness behavior: where do we stand? Brain Behav Immun 15:7–24

Deen CM, Hutchison VH (2001) Effects of lipopolysaccharide and acclimation temperature on induced behavioral fever in juvenile *Iguana iguana*. J Therm Biol 26:55–63

Di Mascio P, Murphy ME, Sies H (1991) Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. Am J Clin Nutr 53:194–200

Diep SK, Westneat DF (2013) The integration of function and ontogeny in the evolution of status signals. Behaviour 150:1015–1044

Doucet SM, Meadows MG (2009) Iridescence: a functional perspective. J R Soc Interface 6:115–132.

Ducrest A-L, Keller L, Roulin A (2008) Pleiotropy in the melanocortin system, coloration and behavioural syndromes. Trends Ecol Evol 23:502–510

Endler JA (1990) On the measurement and classification of colour in studies of animal colour patterns. Biol J Linn Soc 41:315–352

Faivre B, Grégoire A, Préault M, Cézilly F, Sorci G (2003) Immune activation rapidly mirrored in a secondary sexual trait. Science 300:103–103

Feingold KR, Grunfeld C (1992) Role of cytokines in inducing hyperlipidemia. Diabetes 41:97–101

Fitze PS, Tschirren B, Gasparini J, Richner H (2007) Carotenoid-Based Plumage Colors and Immune Function: Is There a Trade-Off for Rare Carotenoids? Am Nat 169:137–144

Fox DL (1979) Biochromy, natural coloration of living things. University of California Press, Berkeley

French SS, DeNardo DF, Moore MC (2007) Trade-Offs between the Reproductive and Immune Systems: Facultative Responses to Resources or Obligate Responses to Reproduction? Am Nat 170:79–89

Galván I, Alonso-Alvarez C (2008) An intracellular antioxidant determines the expression of a melanin-based signal in a bird. PLoS One 3:e3335

Galván I, Alonso-Alvarez C (2009) The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin. Proc R Soc Lond B 276:3089–3097

Galvan I, Solano F (2009) The evolution of eu-and pheomelanic traits may respond to an economy of pigments related to environmental oxidative stress. Pigm Cell Melanoma R 22:339–342

Galván I, Solano F (2015) Melanin Chemistry and the Ecology of Stress. Physiol Biochem Zool 88:352–355

Garant D, Sheldon BC, Gustafsson L (2004) Climatic and temporal effects on the expression of secondary sexual characters: genetic and environmental components. Evolution 58:634–644

Garbe A, Buck J, Hämmerling U (1992) Retinoids are important cofactors in T cell activation. J Exp Med 176:109–117

Geissmann F, Revy P, Brousse N, Lepelletier Y, Folli C, Durandy A, Chambon P, Dy M (2003) Retinoids regulate survival and antigen presentation by immature dendritic cells. J Exp Med 198:623–634

Goodwin TW (1986) Metabolism, nutrition, and function of carotenoids. Annu Rev Nutr 6:273–297

Grill CP, Rush VN (2000) Analysing spectral data: comparison and application of two techniques. Biol J Linn Soc 69:121–138

Halliwell B, Gutteridge JM (2015) Free radicals in biology and medicine. Oxford University Press, Oxford

Halprin KM, Ohkawara A (1966) Glutathione and human pigmentation. Arch Dermatol 94:355–357

Hartley RC, Kennedy MW (2004) Are carotenoids a red herring in sexual display? Trends Ecol Evol 19:353–354

Hegyi G, Török J, Garamszegi LZ, Rosivall B, Szöllősi E, Hargitai R (2007) Dynamics of multiple sexual signals in relation to climatic conditions. Evol Ecol Res 9:905–920

Hong JH, Kim M-J, Park MR, Kwag OG, Lee IS, Byun BH, Leef SC, Leeg KB, Rhee SJ (2004) Effects of vitamin E on oxidative stress and membrane fluidity in brain of streptozotocin-induced diabetic rats. Clin Chim Acta 340:107–115

Hõrak P, Zilmer M, Saks L, Ots I, Karu U, Zilmer K (2006) Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches. J Exp Biol 209:4329–4338

Huang Q-H, Hruby VJ, Tatro JB (1999) Role of central melanocortins in endotoxin-induced anorexia. Am J Physiol-Regul Integr Comp Physiol 276:864–871

Ibáñez A, Polo-Cavia N, López P, Martín J (2014) Honest sexual signaling in turtles: experimental evidence of a trade-off between immune response and coloration in red-eared sliders *Trachemys scripta elegans*. Naturwissenschaften 101:803–811

Introne W, Boissy RE, Gahl WA (1999) Clinical, molecular, and cell biological aspects of Chediak–Higashi syndrome. Mol Genet Metab 68:283–303

Jacot A, Romero-Diaz C, Tschirren B, Richner H, Fitze PS (2010) Dissecting carotenoid from structural components of carotenoid-based coloration: a field experiment with great tits (*Parus major*). Am Nat 176:55–62

Jacquin L, Lenouvel P, Haussy C, Ducatez S, Gasparini J (2011) Melanin-based coloration is related to parasite intensity and cellular immune response in an urban free living bird: the feral pigeon *Columba livia*. J Avian Biol 42:11–15

Janeway CA, Travers P, Walport M, Shlomchik M (2001) Immunobiology. The immune system in health and disease, 5th edition. Garland Publishing, New York

Ji LL (1999) Antioxidants and oxidative stress in exercise. Exp Biol Med 222:283–292

Johnsen A, Delhey K, Andersson S, Kempenaers B (2003) Plumage colour in nestling blue tits: sexual dichromatism, condition dependence and genetic effects. Proc R Soc Lond B 270:1263–1270

Kahn AT, Dolstra T, Jennions MD, Backwell PR (2013) Strategic male courtship effort varies in concert with adaptive shifts in female mating preferences. Behav Ecol 24:906–913

Kemp DJ, Rutowski RL (2007) Condition dependence, quantitative genetics, and the potential signal content of iridescent ultraviolet butterfly coloration. Evolution 61:168–183

Kimball BA, Opiekun M, Yamazaki K, Beauchamp GK (2014) Immunization alters body odor. Physiol Behav 128:80–85

Kopena R, López P, Martín J (2014a) Relative contribution of dietary carotenoids and vitamin E to visual and chemical sexual signals of male Iberian green lizards: an experimental test. Behav Ecol Sociobiol 68:571–581

Kopena R, López P, Martín J (2014b) What are carotenoids signaling? Immunostimulatory effects of dietary vitamin E, but not of carotenoids, in Iberian green lizards. Naturwissenschaften 101:1107–1114

Kopena R, Martín J, López P, Herczeg G (2011) Vitamin E supplementation increases the attractiveness of males' scent for female European green lizards. PLoS One 6:e19410

Krinsky NI, Yeum KJ (2003) Carotenoid–radical interactions. Biochem Biophys Res Commun 305:754–760

Lin SM, Nieves-Puigdoller K, Brown AC, McGraw KJ, Clotfelter ED (2010) Testing the Carotenoid Trade-Off Hypothesis in the Polychromatic Midas Cichlid, *Amphilophus citrinellus*. Physiol Biochem Zool 83:333–342

López P, Gabirot M, Martín J (2009a) Immune challenge affects sexual coloration of male Iberian wall lizards. J Exp Zool Part A 311:96–104

López P, Gabirot M, Martín J (2009b) Immune activation affects chemical sexual ornaments of male Iberian wall lizards. Naturwissenschaften 96:65–69

López P, Martín J (2006) Lipids in the femoral gland secretions of male Schreiber's green lizards, *Lacerta schreiberi*. Z Naturforsch C 61:763–768

Loyau A, Saint Jalme M, Cagniant C, Sorci G (2005) Multiple sexual advertisements honestly reflect health status in peacocks (*Pavo cristatus*). Behav Ecol Sociobiol 58:552–557

Mackintosh JA (2001) The antimicrobial properties of melanocytes, melanosomes and melanin and the evolution of black skin. J Theor Biol 211:101–113

Mader DR (1996) Reptile medicine and surgery. WB Saunders, Philadelphia

Martín J, López P (2006) Vitamin D supplementation increases the attractiveness of males' scent for female Iberian rock lizards. Proc R Soc Lond B 273:2619–2624

Martín J, López P (2009) Multiple color signals may reveal multiple messages in male Schreiber's green lizards, *Lacerta schreiberi*. Behav Ecol Sociobiol 63:1743–1755

Martín J, López P (2010) Multimodal sexual signals in male ocellated lizards *Lacerta lepida*: vitamin E in scent and green coloration may signal male quality in different sensory channels. Naturwissenschaften 97:545–553

Martin M, Le Galliard JF, Meylan S, Loew ER (2015) The importance of ultraviolet and near-infrared sensitivity for visual discrimination in two species of lacertid lizards. J Exp Biol 218:458–465

Mäthger LM, Hanlon RT (2007) Malleable skin coloration in cephalopods: selective reflectance, transmission and absorbance of light by chromatophores and iridophores. Cell Tissue Res 329:179–186

Maynard-Smith J, Harper D (2003) Animal signals. Oxford University Press, Oxford

McGraw KJ (2008) An update on the honesty of melanin-based color signals in birds. Pigm Cell Melanoma R 21:133–138

McGraw KJ, Ardia DR (2003) Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. Am Nat 162:704–712

McGraw KJ, Crino OL, Medina-Jerez W, Nolan PM (2006) Effect of dietary carotenoid supplementation on food intake and immune function in a songbird with no carotenoid coloration. Ethology 112:1209–1216

McGraw KJ, Klasing KC, Dufty AM (2006) Carotenoids, immunity, and integumentary coloration in red junglefowl (*Gallus gallus*). Auk 123:1161–1171

Megía-Palma R (2016) Molecular characterization of lizard parasites and their influence on colour ornaments. PhD Dissertation, Universidad Complutense de Madrid, Madrid

Megía-Palma R, Martínez J, Merino S (2016) Structural- and carotenoid-based throat colour patches in males of *Lacerta schreiberi* reflect different parasitic diseases. Behav Ecol Sociobiol. DOI 10.1007/s00265-016-2205-0

Molnár O, Bajer K, Mészáros B, Török J, Herczeg G (2013) Negative correlation between nuptial throat colour and blood parasite load in male European green lizards supports the Hamilton–Zuk hypothesis. Naturwissenschaften 100:551–558

Molnár O, Bajer K, Török J, Herczeg G (2012) Individual quality and nuptial throat colour in male European green lizards. J Zool 287:233–239

Monaghan P, Metcalfe NB, Torres R (2009) Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol Lett 12:75–92

Montgomerie R (2006) Analyzing colors. In: Hill GE, McGraw KJ (eds) Bird coloration vol 1. Mechanisms and measurements. Harvard University Press, Cambridge, pp 90–147

Moran NA, Jarvik T (2010) Lateral transfer of genes from fungi underlies carotenoid production in aphids. Science 328:624–627

Nathan C, Shiloh MU (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. P Natl Acad Sci USA 97:8841–8848

Navara KJ, Hill GE (2003) Dietary carotenoid pigments and immune function in a songbird with extensive carotenoid-based plumage coloration. Behav Ecol 14:909–916

Olsson MJ, Lundström JN, Kimball BA, Gordon AR, Karshikoff B, Hosseini N, Sorjonen K, Olgart Höglund C, Solares C, Soop A et al. (2014) The scent of disease human body odor contains an early chemosensory cue of sickness. Psychol Sci 25:817-23

Ortonne JP (2002) Photoprotective properties of skin melanin. Br J Dermatol 146:7–10

Palozza P (1998) Prooxidant actions of carotenoids in biologic systems. Nutr Rev 56:257–265

Pérez i de Lanuza G, Font E (2014) Now you see me, now you don't: iridescence increases the efficacy of lizard chromatic signals. Naturwissenschaften 101:831–837

Pérez i de Lanuza G, Font E (2014) Ultraviolet vision in lacertid lizards: evidence from retinal structure, eye transmittance, SWS1 visual pigment genes, and behaviour. J Exp Biol 217:1–11

Peters A, Denk AG, Delhey K, Kempenaers B (2004) Carotenoid-based bill colour as an indicator of immunocompetence and sperm performance in male mallards. J Evol Biol 17:1111–1120

Quinn VS, Hews DK (2003) Positive relationship between abdominal coloration and dermal melanin density in phrynosomatid lizards. Copeia 2003:858–864

Reguera S, Zamora-Camacho FJ, Moreno-Rueda G (2014) The lizard *Psammodromus algirus* (Squamata: Lacertidae) is darker at high altitudes. Biol J Linn Soc 112:132–141

Roulin A (2015) Condition-dependence, pleiotropy and the handicap principle of sexual selection in melanin-based colouration. Biol Rev 91:328-48

Ruiz M, Wang D, Reinke BA, Demas GE, Martins EP (2011) Trade-offs between reproductive coloration and innate immunity in a natural population of female sagebrush lizards, *Sceloporus graciosus.* Herpetol J 21:131

San-Jose LM, Granado-Lorencio F, Sinervo B, Fitze PS (2013) Iridophores and not carotenoids account for chromatic variation of carotenoid-based coloration in common lizards (*Lacerta vivipara*). Am Nat 181:396–409

Santos ES, Scheck D, Nakagawa S (2011) Dominance and plumage traits: meta-analysis and metaregression analysis. Anim Behav 82:3–19

Sasaki M, Horikoshi T, Uchiwa H, Miyachi Y (2000) Up-regulation of tyrosinase gene by nitric oxide in human melanocytes. Pigm Cell Res 13:248–252

Seagle B-LL, Rezai KA, Kobori Y, Gasyna EM, Rezaei KA, Norris JR (2005) Melanin photoprotection in the human retinal pigment epithelium and its correlation with light-induced cell apoptosis. P Natl Acad Sci USA 102:8978–8983

Searcy WA, Nowicki S (2005) The evolution of animal communication: reliability and deception in signaling systems. Princeton University Press, Princeton

Selman C, McLaren JS, Himanka MJ, Speakman JR (2000) Effect of long-term cold exposure on antioxidant enzyme activities in a small mammal. Free Radic Biol Med 28:1279–1285

Shawkey MD, Hill GE (2005) Carotenoids need structural colours to shine. Biol Lett 1:12–124

Slominski A, Tobin DJ, Shibahara S, Wortsman J (2004) Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev 84:1155–1228

Sokal RR, Rohlf FJ (1995) Biometry, 3rd edn. WH Freeman, New York

Spritz RA (2000) Hermansky–Pudlak syndrome and pale ear: melanosome-making for the millennium. Pigm Cell Res 13:15–20

Staszewski V, Boulinier T (2004) Vaccination: a way to address questions in behavioral and population ecology? Trends Parasitol 20:17–22

Surai PF (2002) Natural antioxidants in avian nutrition and reproduction. Nottingham University Press, Nottingham

Sutherland JL, Thompson CF, Sakaluk SK (2012) No Effect of Carotenoid Supplementation on Phytohemagglutinin Response or Body Condition of Nestling House Wrens. Physiol Biochem Zool 85:21–28

Teyssier J, Saenko SV, Van Der Marel D, Milinkovitch MC (2015) Photonic crystals cause active colour change in chameleons. Nat Commun 6:6368

Toomey MB, Butler MW, McGraw KJ (2010) Immune-system activation depletes retinal carotenoids in house finches (*Carpodacus mexicanus*). J Exp Biol 213:1709–1716

Torres R, Velando A (2007) Male reproductive senescence: the price of immune-induced oxidative damage on sexual attractiveness in the blue-footed booby. J Anim Ecol 76:1161–1168

Tsatmali M, Graham A, Szatkowski D, Ancans J, Manning P, McNeil CJ, Graham AM, Thody AJ (2000) α-Melanocyte-Stimulating Hormone Modulates Nitric Oxide Production in Melanocytes. J Invest Dermatol 114:520–526

Uller T, Isaksson C, Olsson M (2006) Immune challenge reduces reproductive output and growth in a lizard. Funct Ecol 20:873–879

Vroonen J, Vervust B, Van Damme R (2013) Melanin-based colouration as a potential indicator of male quality in the lizard *Zootoca vivipara* (Squamata: Lacertidae). Amphibia-Reptilia 34:539–549

Wedekind C (1992) Detailed information about parasites revealed by sexual ornamentation. Proc R Soc Lond B 247:169–174

Wedekind C, Folstad I (1994) Adaptive or nonadaptive immunosuppression by sex hormones? Am Nat 143:936-938

Wu D, Meydani SN (1998) n-3 polyunsaturated fatty acids and immune function. Proc Nutr Soc 57:503–509

Wu G, Fang Y-Z, Yang S, Lupton JR, Turner ND (2004) Glutathione metabolism and its implications for health. J Nutr 134:489–492

Yoshida M, Takahashi Y, Inoue S (2000) Histamine induces melanogenesis and morphologic changes by protein kinase A activation via H2 receptors in human normal melanocytes. J Invest Dermatol 114:334–342

Chapter V

Interindividual differences in morphological traits and sexual signals of male lizards *Lacerta schreiberi* may result in different space use and mating strategies

Renata Kopena, Pilar López and José Martín

Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC. José Gutiérrez Abascal 2, E-28006 Madrid, Spain

Abstract

The movement patterns and space use of animals can reveal the social relationships and mating behavior of a species. In this study we investigated home range size of both males and females and intra- and intersexual overlapping between home ranges and their relationship with sexual signals . We aimed to analyze how the characteristics of the sexual signals of an individual can influence its space use. We found that males with more traits signaling dominance (i.e. more intensive UV-blue throat coloration and relatively greater heads) used larger home ranges that overlap with more individual males and with more home ranges of males with dominance traits, whereas males with smaller home range areas avoided to overlap with these dominant males. Results of home range overlapping of males and females suggested the existence of two different reproductive strategies of males regarding their space use. Males with larger home ranges overlapped with more females, likely trying to increase reproductive success by mating with a higher quantity of females. In contrast, males with smaller home range areas preferred overlapping females with UV-greener backs, so these

males likely tried to increase reproductive success by guarding and mating with fewer females but of higher quality. Females, likely adapting to these male strategies, using larger home ranges overlapped with more males, but females that used small home ranges overlapped with males with high α-tocopherol (= vitamin E) content in chemical signals (i.e., males of high quality). A higher vitamin E content in femoral secretions may indicate good quality and likely high fertility, because vitamin E has important roles in the immune and antioxidant systems in this species. The different space use and mating strategies of males might be two different evolutionary stable strategies, which would provide similar benefits both for mate guarder and dominant males. A similar reproductive success could maintain honesty of signals and, furthermore, the system of multiple sexual signals of this species.

Keywords: Home ranges · Overlapping · Coloration · Chemical signals · Lizards · Alternative strategies

Introduction

Territorial avalaibility of resouces can influence the pattern of space use and the reproductive system of animals (Stamps 1994). If resources are moderately abundant, show moderately patchy distribution or high renewal rates, they can lead to territorial behaviour; while in case of very high or very low resource abundance, high patchiness or low renewal rate of resources, animals do not defend territories and have strongly overlapping home ranges (Pough et al. 2004). Important resources to defend can be, for example, prey availability, refuges, breeding burrows or sunbathing places, but for males the most important resource for territoriality is access to females (Stamps 1994). For males it is worth to be territorial if they possess resources that are attractive for females (Alatalo et al. 1986; Calsbeek and Sinervo 2002). Otherwise, space use of females may constrain males to follow

the movement patterns of females, which may increase the reproductive success of males. If for females it is not worth to defend a territory, in case of conventional sexual roles, home ranges of females are overlapping without any agression or competition among neighbor females (Pough et al. 2004). In monogam mating systems, home ranges of males are strongly overlapping with home ranges of females, especially in the mating season, where males can also do mate guarding. The more that a mating system differs from monogamy to polygyny, the more that males will try to contact with a higher number of females. The larger the surface of the area that males are covering, the higher the number of females that males can meet. This strategy frequently increases the possibility of males to copulate with more females and, consequently, their reproductive success (Haenel et al. 2003). Nevetheless, males with larger home ranges also have a higher possibility to meet more competitor males, leading to intrasexual competition for females. Dominance traits of males,which can be strongly influenced by testosterone, affecting the ontogenetic development or the current condition, can determine the outcome of the agonistic encounters between males. Several conflict situations between males may favor the evolution of dominance signals to help estimate fighting ability in advance to reduce fighting costs (Rohwer 1975; Rohwer 1982). Displays, body size, armaments or, frequently, colorful visual signals (e.g. Tokarz 1985; Sinervo and Lively 1996; Sneddon et al. 2000; Lappin et al. 2006; Henningsen and Irschick 2012), especially melanin-based badges (Santos, Scheck & Nakagawa, 2011; Diep & Westneat, 2013) can have a role as dominance signals.

However, dominance is not always the base of reproductive success (Forsgren 1997; Qvarnström and Forsgren 1998), although production of dominant offspring can be a strategy for females to increase future fitness. However, the expression of dominance traits can be costly. Testosterone can have negative effects on the immune system and increases metabolic rate (e.g. Belliure et al. 2004; Oppliger et al. 2004). Although dominant males frequently can use forced copulation, females can prefer and select sperm of more attractive or healthier males, irrespective of their dominace status, by cryptic mate choice (e.g. Olsson

et al. 1996) against sperm of dominant, but maybe less fertile, males due to their high production of sperm.

As males are closer to monogamy the choosier they are, which may lead to mutual choosiness. In reptiles, frequently some males invest time into mate guarding before and after copulations to ensure paternity, which results in similar movement patterns and home range size than those of the females that males guard. Therefore, mate guarder males need to estimate quality of females, which can be based on body size, condition or coloration of females. However, mate guarding is not in females own interest, because copulation with more males decrease possibility of sterility (Olsson and Shine 1997). But rejection of all mate guarder males can be very costly energetically. Thus for females may be worth to accept being guarded by the more healthy males to avoid steril sperms.

The Iberian or Schreiber's green lizard, which is an endemic species in the Iberian Peninsula, is a large lacertid lizard with strong sexual dichromatism. Adult males have green dorsal coloration with small black spots, yellow chest and ventral coloration and bright blue throat coloration which can extend to mental scales too, while adult females have yellow chest coloration, and white, rarely ligth blue structural throat coloration, however dorsal coloration is more varied, being either green or brown and with variable size and amount of black spots and lines (Marco 2011). Characteristics of sexual coloration of males can be related to morphology, health state, dominance and pairing status. However, these relationships are probably based on different physiological trade-offs (Martín and López 2009). Male lizards use a multimodal signaling system by producing femoral secretions as chemical signals beside the color signals. Femoral secretion containing several lipophilic compounds, especially, large amounts of α-tocopherol (one of the most common forms of vitamin E), which can act as sexual chemical signals (Lopez and Martín 2006a). In the closely related European green lizard (*L. viridis*), which have very similar compounds in secretions (Kopena et al. 2009), females showed preferences for areas scent marked by males with experimentally increased vitamin E levels in their secretions (Kopena et al. 2011).

Furthermore, vitamin E dietary supplementation increased both vitamin E content of femoral secretions and the PHA-induced immune response in male *L. schreiberi* (Kopena et al. 2014b), suggesting that the possible cost of allocating antioxidant vitamin E to secretions may confer reliability to chemical signals of green lizards. Although, females also can signal their quality; we found that females with more intense UV-blue throat coloration have less blood parasites and, furthermore, females with more intense UV-green dorsal coloration have less ticks and larger body size. Females with more yellowish chests also have larger body size, furthermore, bigger females have stronger immune responses. Moreover, different characteristics of female coloration can predict several parameter of reproductive success, such as incubation time of their eggs, hatching success, or size and condition of their hatchlings (Chapter VI). The mating system of *L. schreiberi* is polygynandry (Marco and Pérez-Mellado 1999). Space use behaviour is sedentary (Marco 1996), males and females home ranges overlap with each other (Marco and Pérez-Mellado 1999). According to earlier studies, this species is non-territorial, however it is known that males defend mates (Marco and Pérez-Mellado 1999; Martín and López 2009). Larger males have more and larger mates and, furthermore, a higher mating success, but these do not relate with home range size (Marco and Pérez-Mellado 1999). More dominant males have higher heads and bluer, brighter throats, and more saturated green back coloration (Martín and López 2009). Males show a complete suite of aggressive behaviours, including biting and wrestling, in case of direct fights between two males of similar sizes, chasing, if there is a greater size difference between the males, threaten by chest upraising, and displaying throat coloration (Marco and Pérez-Mellado 1999, personal observation). Also, males may likely use scent-marking with femoral secretions to sign the owner quality to intruders (Lopez et al 1998, Martín and López 2007, Martín et al. 2007). Thereby, body size, sexual coloration and chemical signals might be related with features of the home ranges, and degree of intra- and intersexual overlap, thus, revealing the social and mating system of this species.

In this study, we investigated whether phenotypic morphological traits and characteristics of visual and chemical sexual signals influence space use of both males and females as well as intra- and intersexual overlapping between home ranges, which may determine their mating strategies. We expected individual males with a greater expression of dominance traits (i.e., longer body, bigger head, and more intensive UV-blue throat and greener dorsal coloration) to have larger home ranges that overlap with more males and females. In contrast, males with a lower expression of dominance traits, but with a higher expression of sexual signals signalling a better health (i.e., antioxidant-based chemical signals) may have smaller home ranges, overlapping with less females and males, but overlapped females will be of better quality (i.e., larger size, and greener dorsal, more yellowish chest and more intensive UV-blue coloration).

Materials and methods

Study animals

In April 2010, we captured 25 adult male and 33 female lizards *L. schreiberi* at 'Valle de La Fuenfría' in the Guadarrama mountains (40°44' N, 4°02' W; Madrid Province, Spain). We immediately transferred lizards to ''El Ventorrillo'' field station of the Museo Nacional de Ciencias Naturales (Madrid province, Spain), 5 km from the capture sites. While we took morphological and color measurements (max. 1-2 days), lizards were individually kept in outdoor 51 x 36 x 28 cm PVC terraria containing coconut fiber substratum and rocks for cover. Lizards were fed mealworm larvae (*Tenebrio molitor*), house crickets (*Acheta domesticus)* and common black crickets *(Gryllus assimilis)*, and water was provided *ad libitum*. The photoperiod and ambient temperature were those of the surrounding region.

We measured lizards' body weight before the field study with a digital balance to the nearest 0.1 g (males: mean ± SE=24.2 ± 3.3 g; range=20.0-33.0 g; females: mean ±

SE=24.0 ± 5.6 g; range=14.5-35.0 g) and used a digital caliper to measure snout-to-vent length (SVL) (males: mean ± SE=98.7 ± 6.3 mm; range=87.2-109.7mm; females: mean ± SE=96.5 ± 7.8 mm; range=81.0-110.5 mm) and head size (head height, males: mean ± SE=11.95 ± 0.2 mm; range=9.94-14.29 mm; females: mean ± SE= 10.54 ± 0.20 mm; range=8.62-13.31 mm, head length, males: mean ± SE=24.70 ± 0.40 mm; range=20.63-27.99 mm; females: mean ± SE=21.68 ± 0.35 mm; range=17.96-24.38 mm; head width, males: mean ± SE=13.00 ± 0.20 mm; range=10.77-15.08 mm; females: mean ± SE=11.09 ± 0.16 mm; range=9.45-12.63 mm). We also counted the number of femoral pores of males (mean ± SE=27 ± 1; range=25-30).

We used principal components analysis on the three head variables. First component (PC1) explained 94.12 % of total variance in head size (eigenvalue=2.82, all factor loadings >-0.96). Thus, we used this PC1 instead of the three head measurements in further analyses. We corrected head PC1 and body weight for SVL (all Ln transformed) by using residuals from linear regressions to estimate relative head size and body condition respectively.

Color measurements

We measured reflectance of lizards' coloration (from 300 to 700 nm) using an Ocean Optics USB2000 spectroradiometer equipped with a DT-1000-MINI Deuterium–Halogen light source (Ocean Optics, Inc., Dunedin, FL, USA). This range of wavelengths coincides with the spectral sensitivity measured for other lizard species, including visual perception in the UV range (e.g. Fleishman et al. 1993; Loew et al. 2002). A cylindrical metallic tube mounted on the bifurcated fiber optic probe was used to exclude ambient light and standardize measuring distance (Montgomerie et al. 2006a). The probe was held at a 45^{o} angle to the skin, and reflectance was always measured by the same person (PL). We measured coloration of three visually distinct large patches of coloration ("blue" throat, "yellow" chest, and "green" back in case of males and "white" throat, "yellow" chest, and

"green or brown" back in case of females) at three standardized spots: the middle of the throat (between the last chin shields and the collar; "throat"), the chest (just anterior to the two forelimbs at the middle of the second row of scales prior to the collar; "chest"), and the dorsum (the background coloration in the middle point above the two forelimbs; "back").

We used principal component analyses (PCAs) to summarize mathematically the spectra (Endler 1990; Cuthill et al. 1999; Grill and Rush 2000). The PCA shows all of the information about the shape of reflectance spectra, including bimodal ones like those found in our earlier study of *L. schreiberi* (see Chapter VI) into a few independent PCs (Montgomerie et al. 2006). The first Principal Component (PC1) represents variation in brightness, and subsequent PCs represent combinations of hue and chroma (Endler 1990; Cuthill et al. 1999; Grill and Rush 2000). Moreover, the PCA identifies those sections of the spectrum (wavelength regions) that are contributing to the observed variation, independently of their "importance" in terms of contribution to the total amount of reflectance (Montgomerie et al. 2006). We used different principal component analyses for the different parts of the body (throat, chest and back) including spectra separately for males and females. Furthermore, to confirm the role of carotenoids in chest coloration, we calculated the carotenoid chroma $[(R_{700nm}-R_{450nm})/R_{700nm}]$, which represents relative reflectance around peak absorbance of carotenoids, thus indicating levels of carotenoid pigment content of the integument (Johnsen et al. 2003; Peters et al. 2004). Afterwards, we correlated carotenoid chroma with the PC scores to reveal whether variation in coloration defined by these PC scores could be determined by carotenoid levels. We found significant correlation between in chest PC2 and carotenoid chroma in both gender (males: $r=-0.86$, $P<0.0001$; females $r=0.86$, $P<0.0001$) showing that chest yellow coloration is carotenoid dependent both in males and females.

Determination of α-tocopherol content in femoral secretions

Femoral secretion of males was collected into glass vials with glass inserts, which were closed with Teflon-lined stoppers and stored at -20 ºC. Samples were analyzed with a gas chromatograph-mass spectrometer (Finnigan-ThermoQuest GC-MS Trace 2000, Thermo-Quest Corporation, Austin, Texas) equipped with a Supelco-Equity-5 (Bellefonte, PA) column temperature programmed (50–280 ºC at 5 ºC/min and 280 ºC for 30 min). Identification of compounds was performed by comparison of the obtained mass spectra with those in the NIST/EPA/NIH (NIST 02, www.nist.gov) library, and later confirmed with authentic standards (see López and Martín 2006 for details of analyses and chemical compounds of secretions in *L. schreiberi*). The relative amount of α-tocopherol was determined as the percent of the total ion current (TIC) area, transforming it for calculations using the Aitchison's formula: [Zij=ln(Yij/g(Yj)], where Zij is the standardized peak area i for individual j, Yij is the peak area i for individual j, and g(Yj) is the geometric mean of all peaks for individual j (Aitchison 1986).

Field study

After morhological data collection, we individually marked animals by using long lasting nail polish, with a combination of three small color marks placed on three body locations (head, back, base of the tail). After the observations, animals lose these marks naturally and nail polish did not cause any health damage for them. We released animals exactly at the same place where we had collected them one or two days before. The area where animals had been captured was a half-opened sunny patch inside a *Pinus sylvestris* forest, with shrubs such as *Rubus ulmifolius*, *Juniperus communis* and *Cytisus scoparius.* This area is more or less closed for the animals because the surrounding pine forest was a suboptimal habitat (i.e., it had less light, less refuges and probably less food), and from the sunny patch the closest optimal open area was at least 500m away, where we never collected animal from. Furthermore, we had never seen adult lizards in the patch surrounding the pine

forest (RK, five years long personal observation). The observation period lasted for 30 days, when the weather let the animals to be active. Every day from 900h to 1900 the same observer (RK) checked by using binoculars the localization of sighted marked lizards every hour, identifying individuals by their color code. The observation area was patchy with shrubs, rocks and weeds, where the detectability can differ. Thus, we compensated this difference by expended different amount of searching time in different areas as to reach similar probabilities of detection. The location of lizards was determined by using a GPS (Garmin) using a fixed transect in every hour. To ensure independence of data point, we collected only one data point per hour per animal.

Home range size and overlapping calculation

Animal's home range are defined most commonly as the smallest area where the animal can be found with a 95% probability (White and Garrott 1990). Thus, we used 95% kernel-home range estimates to assess home range size (Fig. 1). We applied bivariate normal distributed kernels calculated with the adehabitatHR R package (Calenge 2015) in the R statistical environment (R Core team 2016). All individuals with less than 5 localization points throughout the study period were excluded from further analyses. A total of 58 animals were marked, and, thus, we were left with 42 individuals (23 males and 19 females) after the exclusion.

We estimated overlap of home ranges by calculation of the probability to find the animal "j" in the home range of animal "i" which was determined by utilization of the distribution overlap index (UDOI) (Fieberg and Kochanny 2005) estimated with the adehabitatHR R package (Calenge 2015). We considered two individuals to have overlapping home ranges, if the probability of finding them in each other range was higher than 0.25. For each individual, we calculated the median value of variables measured in overlapping conspecifics (SVL, body condition, relative head PC, colour PCs, femoral

pores*, α-tocopherol content of femoral secretion*; variables signed with an asterisk were measured only in males).

Statistical Methods

Initially, we used an unbiased random forest (Breiman 2001) to pre-select explanatory variables (SVL, body condition, relative head PC, color PCs, femoral pores*, α-tocopherol content of femoral secretion*; variables signed with an asterisk were measured only in males) by estimating variable importance prior to all modelling with the party R package (Hothorn et al. 2015). Random forests have high predictive accuracy with "small n, large p" datasets even in case of highly correlated variables (Strobl et al. 2008). Here, we used the AUC based permutation variable estimating the algorithm suggested by Janitza et al. (2013). We chose the first three most influential variables to include into regression modelling, and performed a backward stepwise selection procedure to establish the best fitting general lineal models (BS-GRM with STATISTICA 7.0; StatSoft, Inc, Tulsa, OK, USA). We also used conditional and regression trees (CART) models to check for potential confounding interactions using all potential explanatory variables with the party R package (Hothorn et al. 2015). Because CART models tend to overfit data in case of low sample sizes (Jin 2013), we determined the size of trees by stopping criteria of tree building to avoid overfitting. We used default setting in the party R package (mincriterion=0.95, that is 1 – α, stopping tree building when no p value is below a pre-specified α-level; minimum number of cases within each intermediate (minsplit=10) and terminal node (minbucket=5). We also used BS-GRMs on variables highlighted by CART models to support the results. However, the regression method can confirm our results only when the piecewise-constant surface approximates the smooth regression surface, but strongly jagged responses are detectable rather by CART models (Trevor and Tibshirani 1990). We made pairwise correlations of significant independent variables and all variables to confirm roles and relationships of traits.

Table 1 Results of Principal Component Analyses of different body part coloration of male and female *L. schreiberi*.

		% of the variation	Eigen-value	PC scores		Relationship of the variables with PC scores		Interpretation
Males				mean	range	*positive*	*negative*	
throat	PC1	71.33	57.78	1.68	-9.13–12.55	-	300-700 nm	Lower PC scores---> brighter throat
	PC2	17.47	14.15	0.46	-8.00–6.87	300-540 nm	545-700 nm	Higher PC scores--->more saturated UV-blue throat
	PC3	8.44	6.84	0.23	-3.06–4.62	410-585 nm	300-405 nm 590-700 nm	Higher PC scores--->higher relative amount of short and middle wavelengths comparing to very short and long wavelengths
	Total	97.25	78.77					
chest	PC1	64.38	52.15	-0.46	-12.51–9.36	-	300-700 nm	Lower PC scores--->brighter chest
	PC2	22.51	18.24	-0.80	-8.37–6.86	300-505 nm	510-700 nm	Lower scores--->more saturated yellow chest
	PC3	7.92	6.41	0.41	-4.68–4.57	400-570 nm	300-395 nm 575-700 nm	Lower scores--->higher relative amount of very short (UV) and long wavelengths comparing to short and middle wavelengths (i.e. more UV-yellow chest)
	Total	94.81	76.80					
back	PC1	64.47	52.22	0.54	-16.21–7.73	-	300-700 nm	Lower PC scores--->brighter back
	PC2	17.04	13.80	0.02	-6.50–4.70	300-435 nm	440-700 nm	Lower PC scores--->more saturated green back
	PC3	11.36	9.02	-0.05	-6.06–6.30	390-525 nm	300-385 nm 530-700 nm	Higher PC scores---> higher relative amount of short wavelengths comparing to very short (UV) and middle-long wavelengths (i.e. less UV-green back)
	Total	92.86	75.22					
Females								
throat	PC1	86.45	70.02	-0.75	-13.42–18.38	-	315-700 nm	Lower PC scores---> brighter throat
	PC2	9.73	7.88	-0.29	-4.69–4.36	430-640 nm	300-425 mm 645-700 nm	Higher PC scores--->more saturated short (blue) and middle-long (green-yellow) wavelength comparing to very short (UV) and very long wavelengths
	PC3	2.24	1.82	-0.22	-2.19–1.85	300-560 nm	565-700 nm	Higher PC scores--->higher relative amount of short and middle wavelengths comparing to long wavelengths (i.e. more UV-blue throat)
	Total	98.42	79.72					
chest	PC1	80	64.8	-1.85	-16.44–14.24	-	300-700 nm	Lower PC scores--->brighter chest
	PC2	11.72	9.49	-0.13	-5.29–5.84	435-670 nm	300-430 nm 675-700 nm	Higher scores--->more saturated yellow chest
	PC3	5.29	4.29	0.27	-3.00–4.28	300-325 nm 370-525 nm	330-365 nm 530-700 nm	Lower scores--->higher relative amount of UV and middle-long wavelengths comparing to certain part of very short and short wavelengths (i.e. more UV-yellow chest)
	Total	97.01	78.58					
back	PC1	76.44	61.92	-1.14	-17.88–8.31	-	300-700 nm	Lower PC scores--->brighter back
	PC2	13.01	10.54	0.16	-7.26–5.60	300-430 nm	435-700 nm	Lower PC scores--->more saturated green or brown back
	PC3	6.11	4.95	-0.04	-4.28–3.67	340-600 nm	300-335 nm 605-700 nm	Higher PC scores---> higher relative amount of short-middle wavelengths comparing to very short and long wavelengths (i.e. more UV-green back)
	Total	95.57	77.41					

Results

Home range sizes

Median of females' home range size was 1268m^2 (range=55-4336 m^2) and median of males' home range size was 1712m^2 (range=483-13007 m^2). We did not find significant differences in home range sizes between sexes (Mann-Whitney U test; U=155.50, p=0.18).

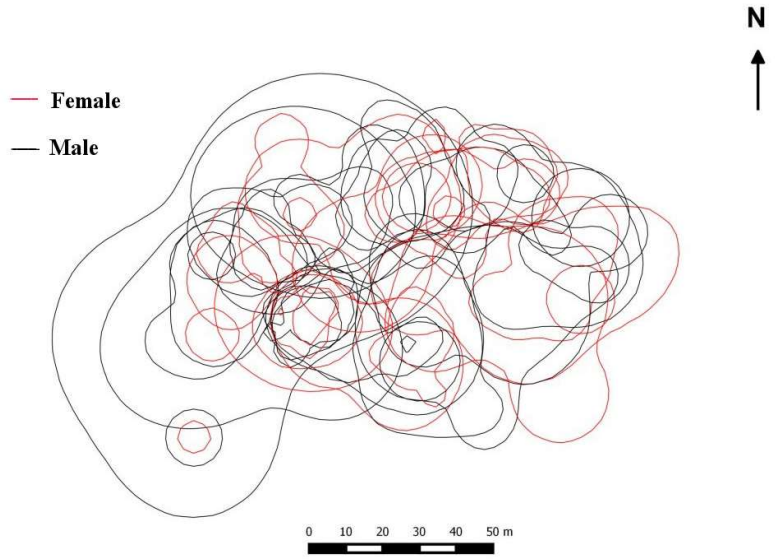

Fig 1 Kernels of male (black line) and female (red line) lizards.

Relationships of male traits and home range size

The random forest analysis showed that the first three most important variables related with home range size of males were relative head size, throat PC2 coloration and α-tocopherol. α-tocopherol was eliminated out by BS-GRM, but relative head size (Fig. 2a) and throat PC2 (Fig. 2b) remained significant after the elimination processes (BS-GRM, model: R^2=0.61, $F_{2,14}$=13.32, p<0.001; relative head PC: β=-0.50, t=-3.18, p=0.007; throat PC2: β=0.59, t=3.74, p=0.002) (Fig. 2b,c). Thus, males with more UV-blue throats (300-540 nm) and relatively greater heads had larger home range areas. The CART model also showed

that males with more intensive UV-blue coloration and relatively greater heads had larger home range areas (Fig.2a).

Relationships of female traits and home range size

Random forest did not find any important variable in the relationship of morphological and color traits with home range size of females. The CART model showed that females with lower values of dorsal PC2 coloration (lower values of 430-700 nm, i.e., lower back saturation) had smaller home ranges (Fig.3), but the linear regression did not reach significance (R^2=0.20, $F_{1,13}$=3.16, p=0.099).

Relationships of male home range size and morphological and coloration traits of overlapped males

Males home ranges overlapped a median of 5 males (range = 1-17). Random forest showed that the first three most important explaining variables for log home range size were the number of overlapped males, throat PC3 and throat PC2 coloration of overlapped males. Backward stepwise regression found that number of overlapped males and thPC2 were significant (BS-GLM, model: R^2=0.67, $F_{2,20}$=23.29, p<0.001; number of overlapped males: β=0.80, t=6.54, p<0.001; overlapped male thPC2: β=0.31, t=2.50, p=0.021) (Fig.4b) but throat PC3 was eliminated. Also, the CART method found that the number and throat PC2 of overlapped males influenced home range size of males (Fig. 4a). So, the larger a male home range was, the more conspecific males it will overlap. Moreover, a larger HR means that the median thPC2 of overlapped conspecific males was also higher. In other words, males with more UV-bluish throats did not avoid each other, but they were avoided by males with less UV-bluish throats.

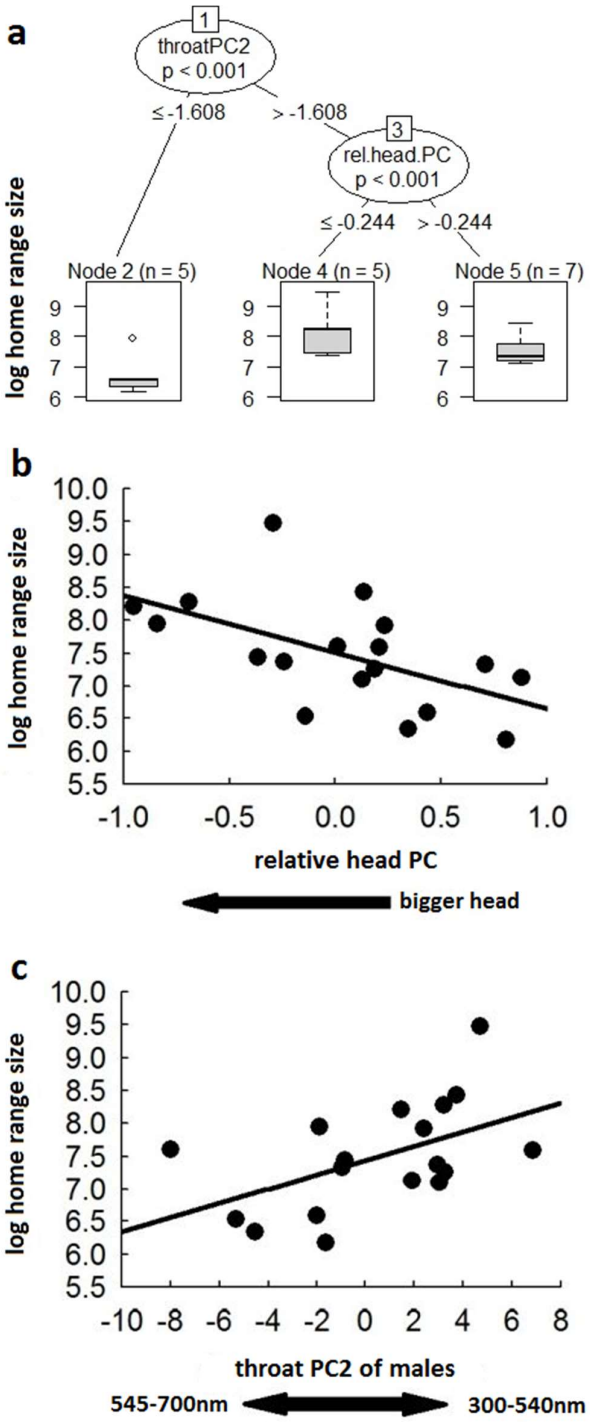

Fig. 2 Relationships of log home range size, throat PC2 and relative head size of male lizards with CART method (**a**) and regression (**b, c**). Boxplots of terminal nodes show median, upper and lower quartiles and range of log home range size of males (**a**).

Relationships of male home range size and morphological and coloration traits of overlapped females

Males' home ranges overlapped a median of 4 home ranges of females (range = 1-13). Random forest model showed a pattern of importance of some variables. Among the first three variables related with home range size of males, number of overlapped females, chPC2 and SVL of overlapping females, only the first variable was significant in a GRM (BS-GRM, model: R^2=0.56, $F_{1,21}$=29.17, p<0.001; number of overlapped females: β=0.76, t=5.42, p<0.001). The CART model showed that males with larger home ranges overlapped with more females, but males that had smaller home ranges overlapped with less females but these females had UV-greener back coloration (i.e., higher values of back PC3) (Fig 5a). Using these two variables in the GRM model, we found significant results for both (BS-GRM, model: R^2=0.67, $F_{2,20}$=23.88, p<0.001; number of overlapped females: β=0.26, t=6.84, p<0.001; dPC3 of overlapped females: β=-0.37, t=-2.87, p=0.009; Fig. 5b).

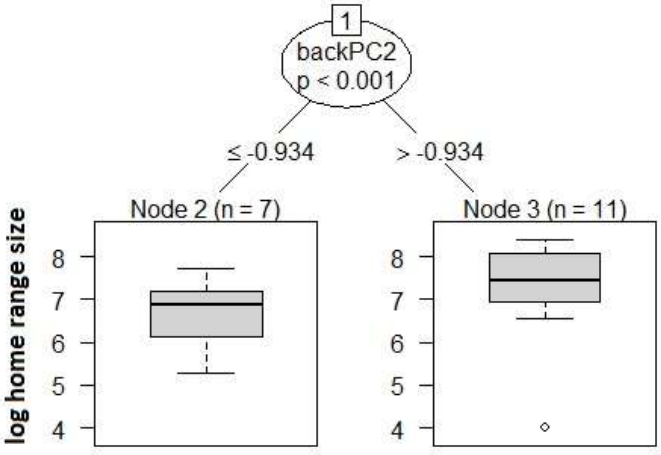

Fig. 2 Relationship of log home range size and back PC2 of females. Boxplots of terminal nodes show median, upper and lower quartiles and range of log home range size of females.

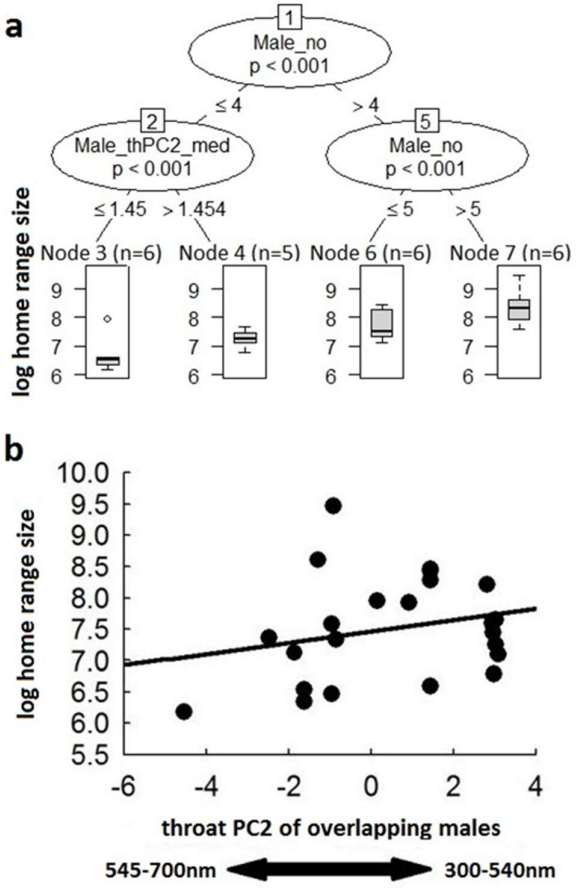

Fig 3 Relationships of log home range sizes and number and throat PC2 of overlapping males with CART method (**a**) and regression (**b**). Boxplots of terminal nodes show median, upper and lower quartiles and range of log home range size of males (**a**).

Relationships of female home range size and traits of overlapped females

Home ranges of females overlapped a median of 3 home ranges of other females (range = 0-9). Random forest model did not find any important variables. The CART model (Fig. 6a) showed that females with smaller home ranges overlapped less females, but females overlapping more than two females had smaller home ranges if overlapping females had higher values of backPC3 (i.e. greener) (Fig. 6a). BS-GRM found that only the number of overlapping females was significant (stepwise GRM, model: R^2=0.35, $F_{1,16}$=10.29, p<0.001; number of overlapping females: β=0.33, t=3.21, p=0.005).

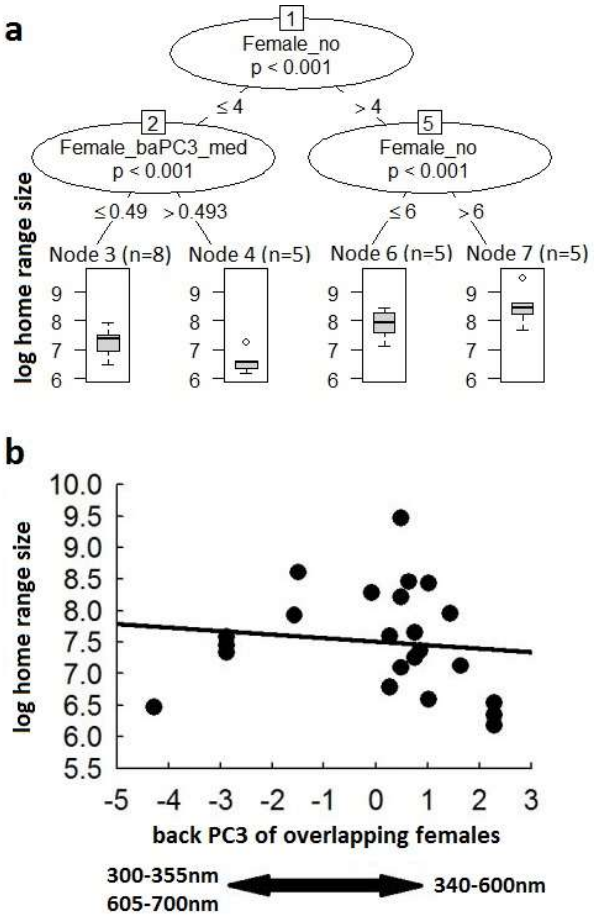

Fig. 4 Relationships of log home range size of males and number and back PC3 of overlapping females with CART method (**a**) and regression (**b**). Boxplots of terminal nodes show median, upper and lower quartiles and range of log home range size of males (**a**).

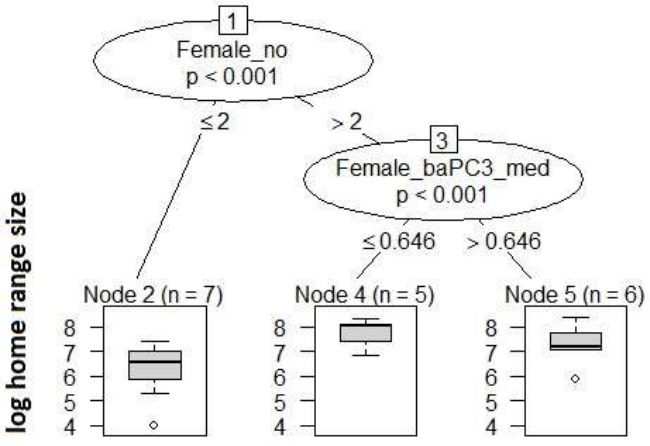

Fig. 5 Relationships of log home range size and number and traits of overlapping females with CART method. Boxplots of terminal nodes show median, upper and lower quartiles and range of log home range size of females.

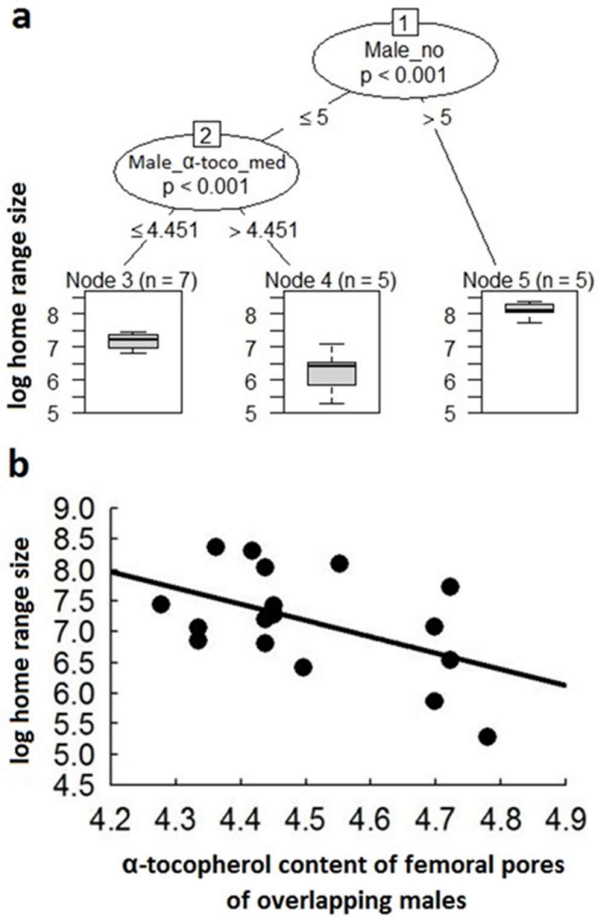

Fig. 6 Relationships of log home range size of females and number and α-tocopherol content of femoral secretion of males with CART method (**a**) and regression (**b**). Boxplots of terminal nodes show median, upper and lower quartiles and range of log home range size of females (**a**).

Relationships of female home range size and traits of overlapped males

Home ranges of females overlapped a median of 5 home ranges of other males (range =1-17). Random forest did not find any important variables. The CART method showed that females who had larger home range areas overlapped more males, while females with the smallest home ranges overlapped with less males, but these males had a higher content of α-tocopherol in their femoral secretion, but in case of lower α-tocopherol content females increased their home range size (Fig. 7a). GRM confirmed these results (BS-GRM, model: R^2=0.62, $F_{2,14}$=13.91, p<0.001; number of overlapped males: β=0.18, t=4.17, p<0.001; α-tocopherol of overlapped males: β=-2.94, t=-3.60, p=0.003; Fig. 7b).

Correlations of male and female traits

We investigated correlations between male traits related with space use of both gender (relative head size, thPC2 and α-tocopherol content of femoral secretion) and variables that can have a role as male quality traits (all color PCs, number of femoral pores, α-tocopherol content of femoral secretion, relative head size, SVL, condition). We found that relative head PC correlated positively with α-tocopherol content in femoral secretion ($F_{1,15}$=5.29, r=0.51, p=0.037), and negatively with body condition ($F_{1,16}$=6.66, r=-0.54, p=0.02). Thus, males with relative bigger head had less α-tocopherol in femoral secretions but had better body condition. The content of α-tocopherol in femoral secretion correlated significantly and positively with number of femoral pores ($F_{1,15}$=9.00, r=0.60, p=0.009) and negatively with values of dPC3 coloration ($F_{1,15}$=9.40, r=-0.62, p=0.008), thus males with more α-tocopherol in femoral secretion also had more femoral pores and greener dorsal coloration. Other correlations were not significant (p>0.05 for all).

We also examined correlations between female traits related their space using and other quality traits (all color PCs, number of femoral pores, relative head size, SVL, condition). We found a significant negative correlation between values of back PC2 and chest PC2 ($F_{1,13}$=4.80, r=-0.52, p=0.047), so females with more saturated back had more saturated yellow chests. Furthermore, values of back PC3 correlated positively with SVL ($F_{1,13}$=6.50, r=0.58, p=0.024) and throat PC3 ($F_{1,13}$=4.96, r=-0.52, p=0.044), so females with greener backs were bigger (at least in this small sample) and had more intense UV-bluish throats. Other correlations were not significant (p>0.05 for all).

Discussion

We found that males with dominance traits had larger home ranges that overlapped with more males and that these overlapped males had more dominance traits, whereas males having smaller home ranges area avoided these dominant males. Results of males

overlapping females draw clearly two different mating strategies as suggested by their different space use strategies; males with larger home ranges overlapped more females, likely trying to increase their reproductive success by mating with more females. However, males having smaller home ranges overlapped with less females, but these females had UV-greener backs, so males likely tried to increase their reproductive success by choosing females of higher quality. Females, likely adapting to these male strategies, having larger home ranges overlapped with more males, but females overlapping males with high content of α-tocopherol in femoral secretion had smaller home ranges.

We found that males with relative bigger head size or more intense UV-blue throat coloration had larger home ranges. Moving over a larger area implies overlapping home ranges of more males and, likely, increasing the frequency of agonistic encounters between males. The consequence of a bigger head is a stronger bite (Perry et al. 2004; Huyghe et al. 2009) resulting in higher fighting ability. Hereby, males with bigger heads should have advantages in agonistic encounters and can be more dominant. Factors influencing relative head size are unclear, but testosterone can have an important role on this in early life development; however, there is no direct evidence (Vitt and Cooper Jr 1985; Sassoon et al. 1987; Perry et al. 2004; Huyghe et al. 2009). Furthermore, relative head size correlated with body condition providing even a better condition for aggressive encounters. Intensive UV-blue coloration of throat is a basic dominant status signal in Iberian and also in European green lizards (Martín and López 2009b; Bajer et al. 2011). Throat coloration is structural and melanin-based; decreasing melanin content decreases UV and blue color of skin (Megía-Palma et al 2016). Testosterone can increase melanin-based social badges (Evans et al. 2000; Gonzalez et al. 2001) and, furthermore, elicits more aggressive behavior (Wingfield et al. 1987), which frequently associates with a higher activity and greater home range size (DeNardo and Sinervo 1994). However, it is not proven that the throat patch is a testosterone directed social signal, but effect of testosterone would explain the higher movement activity and the higher dominant status. Furthermore, intensity of coloration of this patch did not correlate with any morphological traits which could provide advantages in fights (such as

relative head size, condition or body length) that increase plausibility of an internal effect on dominance in males with more intensive throat coloration.

Males with larger home ranges overlapped males that had more intense UV-blue throats, but males with smaller home ranges avoid them. Interestingly, we did not find the same results for relative head size, which may support that males discriminate dominance and fighting ability based on throat coloration. Reason of avoiding males with more intense UV-blue coloration may be the elicited higher level of stress by aggression because probability of being attacked can be higher.

Males showed differences in strategies when overlapping with female home ranges. Males with larger home ranges overlapped with several females, whereas males with smaller home ranges overlapped with less females, but these females had more UV-green back colorations. An earlier study on *L. schreiberi* showed that, although home range size did not relate with mating success, the number of overlapping with females did correlate with number of obtained matings (Marco and Pérez-Mellado 1999). More contact with more females usually increases reproductive success in lizards (Haenel et al. 2003), especially in case of promiscuous species. Although dominant males with bigger heads or more aggressive behavior can get forced-copulations with females, females do not always prefer these dominant males (Qvarnström and Forsgren1998), and cryptic mate choice can allow to females to have mate preferences (Olsson and Madsen 1998). Males with less dominant traits, had smaller home ranges and rather invested in mate guarding of a good quality female. The smaller their home ranges were, the greener back coloration the overlapping females had. This could suggest a stricter mate guarding strategy. In other study, we found that females with more UV-green back coloration have larger body size and less parasites, and, furthermore, have faster developing embryos in eggs. Moreover, in this small sample, females with greener dorsal coloration had longer body size and more intense UV and bluish throat coloration. In an earlier study, we found that females with longer body size had better PHA-induced immune response and lay more eggs. More intense UV-blue throat coloration signals less blood parasites and hatchlings with better condition. These offspring have

advantage by their initial good condition and maybe they inherited from mothers a better parasite resistance and possible faster growth rate (Chapter VI). Mating with a high quantity of females or with less females but of higher quality may lead to similar reproductive success. Male followers of the strategy of "female quantity" may have some offspring from several females and they would not waste time in mate guarding, but females can use cryptic mate choice and sperm competition. A larger home range requires more energy for movement and for fights, and, furthermore, a high number of copulations require a high quantity of sperm and antioxidant rich semen. Testosterone, however, can have positive effects on quantity and motility of sperm (Depeiges and Dacheux 1985; Anjum and Reddy 2015), can increase oxidative stress (Alonso-Alvarez et al. 2007) damage sperm (Tremellen 2008) and enhance antioxidant requirements. In contrast, males with smaller home ranges may invest a lot of time and energy into mate-guarding; however, the probability of sperm competition may be lower, and, furthermore they can afford to produce less quantity of sperm but of higher quality.

The CART model showed that home range size of females was dependent of the saturation of their back coloration. However, nor random forest neither linear regression confirmed it. Smaller home range size may be concomitant with a lower predation risk and lower stress, which can allow increasing back color saturation in females of this species (Chapter VI). Females with more saturated backs have more saturated yellow chests too, which can support they are affected by lower stress level because carotenoids react with stress provoking free radicals (Krinsky and Yeum 2003).

We found with the CART model that females with the smallest home range areas overlapped with less than two females, but if they overlapped more than two females, females overlapping females with more UV-green back coloration had smaller home ranges than females overlapping females with less UV-green back. However random forest did not found any important variables and GRM found that only the number of overlapping females was significant. A possible reason for this result can be that these females reduced home range areas because females with more UV-green back coloration (i.e., of higher quality)

can attract high quality males into their home ranges. Therefore, it may be rather worth to overlap with these females and try to copulate with their high-quality males than invest energy moving on larger home range areas to try to copulate with a high number of males.

Home ranges of females were influenced by behavior of males. We found that females having larger home ranges overlapped with more males, suggesting more mating possibilities, but for females that overlapping with less males, the smaller their home ranges were the higher the α-tocopherol content of femoral secretion of overlapping males was. Interest of females in a polygynic system is mating with as many and good quality males as they can do. Overlapping with more males can imply more mating and a high possibility for cryptic mate choice to select the best sperms from the best males. But if females are chosen by a mate guarder male and avoiding to be guarded is too energetically costly, females can accept this male as a partner. But losing the advantage of cryptic mate choice, females have to estimate quality of males and importance of sperm condition is higher in a mate guarding situation due to the low number of mating partners. Appropriate estimate of sperm condition increases possibility of success in reproduction. Sperm condition is strongly influenced by antioxidants, α-tocopherol has an important role to avoid infertility by maintaining sufficient quantity, viability and motility of spermatozoa with unfragmented DNA and free-radical resistant, polyunsaturated fatty acid rich plasma membrane (e.g., Suleiman et al. 1996; Surai et al. 1997; Keskes-Ammar et al. 2003; Greco et al. 2005; Eid et al. 2006). Females can prefer males with higher α-tocopherol content in femoral secretion to ensure their own reproductive success and increase attractiveness of their offspring. In case of lower α-tocopherol content, females used larger home range areas, maybe for increasing mating opportunities with more males. In an earlier study, we found in female *L. viridis* a preference for areas scent-marked by males with more α-tocopherol in secretions (Kopena et al. 2011). This may support that α-tocopherol may have an important role in mate choice of *L. schreiberi* too.

Correlations between male traits supported the use of two different mating strategies. A dominance trait such as relative head size was correlated positively with body

condition but negatively with signals attractive for females - such as α-tocopherol and its correlating femoral pores number, and intensity of UV-green back coloration. A higher number of femoral pores can produce more secretions and, thus, made a stronger signal. A high percent of α-tocopherol coupling to a large amount of femoral secretion may signal more efficiently the high quality of males. Back coloration is carotenoid based which can be supported by α-tocopherol in *L. schreiberi* (Chapter II but see Chapter IV). More carotenoids in skin can confirm a better quality of sperm due to the higher amount of available antioxidants. In great tits (*Parus major*), males with more colourful carotenoid-based breast plumage have higher sperm motility and swimming ability, and decreased levels of sperm lipid peroxidation (Helfenstein et al. 2010). However, our results do not fit exactly to earlier results in traits of dominant and mate guarder males. In a previous study, dominant males have higher heads both in absolute and relative meaning, brighter but more intensive UV-blue throats, and darker and greener backs, whereas mate guarder males have darker but more intensive UV-blue throats and more yellow chests (Martín and López 2009b). The most important difference may be intensity of UV-blue throat of males that are dominant and attractive traits at the same time in the earlier study, whereas in the current study, it is rather a dominant trait alone. However UV-blue intensity of male throats is not related with dominant traits nor with attractive traits, only with home range size. UV, but not blue intensity of throat was both a dominant and attractive signal also in the closely related *L. viridis* (Bajer et al. 2010; Bajer et al. 2011). Maybe this double role can disrupt correlations, however, movement on larger home range areas contradicts with mate guarding, except if they guard females only for short periods.

Different reproductive strategies of males and behavioural adaptation of females to these strategies can explain the similar home range sizes of both sex and support the existence of mixed evolutionary stable strategies (mixed ESS; Maynard Smith 1982; Számadó 2000). Moreover, attractive traits for females may change between years due to different environmental conditions, which may possible lead to flexible female preferences (Chapter IV), but the basic dominant-attractive strategies of males can be permanent resulting in similar reproductive success for the males. Using of evolutionary stable

alternative strategies in reproduction is frequent but not universal in the animal kingdom (Gadgil 1972; Emlen 1997; Moczek & Emlen 2000; Tuttle 2003). It is especially usual in polymorphic species where animals with distinct traits apply distinct alternative tactics (Taborsky et al. 2008). However, in males of *L. schreiberi*, continuous traits apparently have a role in alternative mating strategies, which has been rarely found in previous studies with other animals. For example, yellow warbler (*Dendroica petechial*) males with brighter plumage have highest growth-rated nestling despite of lowest nest visit ranges, which can be explained by a better quality territory, whereas duller males have high nest visit ranges but their territories are of poor quality (Studd and Robertson 1985).

Although our results are based on a small part of a population, they can provide an exciting direction for further investigation to understand the mating and reproductive system of *L. schreiberi*. The role of ESS needs confirmation by genetic researches and long-term repeated field observations to prove the stability of the social system and equality of fitness of both strategies.

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Ethical standards

The experiments enforced all the present Spanish laws and were performed under license (permit number: 10/142790.9/11) from the Environmental Organisms of Madrid Community where they were carried out.

References

Alatalo RV, Lundberg A, Glynn C (1986) Female pied flycatchers choose territory quality and not male characteristics. Nature 323:152–153

Alonso-Alvarez C, Bertrand S, Faivre B, Chastel O, Sorci G (2007) Testosterone and oxidative stress: the oxidation handicap hypothesis. Proc R Soc Lond B 274:819-825

Anjum MR, Sreenivasula RP (2015) Recovery of lead-induced suppressed reproduction in male rats by testosterone. Andrologia 47:560-567

Bajer K, Molnár O, Török J, Herczeg G (2010) Female European green lizards (*Lacerta viridis*) prefer males with high ultraviolet throat reflectance. Behav Ecol Sociobiol 64:2007–2014

Bajer K, Molnár O, Török J, Herczeg G (2011) Ultraviolet nuptial colour determines fight success in male European green lizards (*Lacerta viridis*). Biol Lett 7:866–868

Belliure J, Smith L, Sorci G (2004) Effect of testosterone on T cell-mediated immunity in two species of Mediterranean lacertid lizards. J Exp Zool A 301:411–418

Breiman L (2001) Random forests. Mach Learn 45:5–32

Calenge C (2011) Home range estimation in R: the adehabitat HR package. Office national de la classe et de la faune sauvage, Saint Benoist, France.

Calsbeek R, Sinervo B (2002) Uncoupling direct and indirect components of female choice in the wild. P Natl Acad Sci USA 99:14897–14902

Cuthill IC, Bennett ATD, Partridge JC, Maier EJ (1999) Plumage reflectance and the objective assessment of avian sexual dichromatism. Am Nat 153:183–200

DeNardo DF, Sinervo B (1994) Effects of steroid hormone interaction on activity and home-range size of male lizards. Horm Behav 28:273-287

Depeiges A, Dacheux JL (1985) Acquisition of sperm motility and its maintenance during storage in the lizard, *Lacerta vivipara*. J Reprod Fertil 74:23-27

Diep SK, Westneat DF (2013) The integration of function and ontogeny in the evolution of status signal. Behaviour 150:1015–1044

Eid Y, Ebeid T, Younis H (2006) Vitamin E supplementation reduces dexamethasone-induced oxidative stress in chicken semen. Br Poult Sci 47:350–356

Emlen DJ (1997) Alternative reproductive tactics and male-dimorphism in the horned beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). Behav Ecol Sociobiol 41:335-341

Endler JA (1990) On the measurement and classification of colour in studies of animal colour patterns. Biol J Linn Soc 41:315–352

Evans MR, Goldsmith AR, Norris SR (2000) The effects of testosterone on antibody production and plumage coloration in male house sparrows (*Passer domesticus*). Behav Ecol Sociobiol 47:156–163

Fieberg J, Kochanny CO (2005) Quantifying home-range overlap: the importance of the utilization distribution. J Wildl Manag 69:1346–1359

Fleishman LJ, Loew ER, Leal M (1993) Ultraviolet vision in lizards. Nature 365:397

Forsgren E (1997) Female sand gobies prefer good fathers over dominant males. Proc R Soc Lond B 264:1283–1286

Gadgil M (1972) Male dimorphism as a consequence of sexual selection. Am Nat 106:574-580

Gonzalez G, Sorci G, Smith LC, Lope F (2001) Testosterone and sexual signalling in male house sparrows (*Passer domesticus*). Behav Ecol Sociobiol 50:557–562

Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J (2005) Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. J Androl 26:349–353

Grill CP, Rush VN (2000) Analysing spectral data: comparison and application of two techniques. Biol J Linn Soc 69:121–138

Haenel GJ, Smith LC, John-Alder HB (2003) Home-range analysis in *Sceloporus undulatus*. II. A test of spatial relationships and reproductive success. Copeia 2003:113-123.

Hastie TJ, Tibshirani RJ (1990) Generalized additive models. Chapman and Hall, London

Helfenstein F, Losdat S, Møller AP, Blount JD, Richner H (2010) Sperm of colourful males are better protected against oxidative stress. Ecol Lett 13:213-222

Henningsen JP, Irschick DJ (2012) An experimental test of the effect of signal size and performance capacity on dominance in the green anole lizard. Funct Ecol 26:3-10

Hothorn T, Hornik K, Strobl C, Zeileis A (2015) party: A Laboratory for Recursive. URL http://CRAN.R-project.org/package=party. R package version 1.0-25. Accessed 04 November 2015

Huyghe K, Herrel A, Adriaens D, Tadić Z, Van Damme R (2009) It is all in the head: morphological basis for differences in bite force among colour morphs of the Dalmatian wall lizard. Biol J Linn Soc 96:13–22

Janitza S, Strobl C, Boulesteix A-L (2013) An AUC-based permutation variable importance measure for random forests. BMC Bioinformatics 14:119

Keskes-Ammar L, Feki-Chakroun N, Rebai T, Sahnoun Z, Ghozzi H, Hammami S, Zghal K, Fki H, Damak J, Bahloul A (2003) Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. Arch Androl 49:83–94

Kopena R, López P, Martín J (2009) Lipophilic compounds from the femoral gland secretions of male Hungarian green lizards, *Lacerta viridis*. Z NaturforschC 64:434–440

Kopena R, Martín J, López P, Herczeg G (2011) Vitamin E supplementation increases the attractiveness of males' scent for female European green lizards. PLoS One 6:e19410

Krinsky NI, Yeum KJ (2003) Carotenoid–radical interactions. Biochem Biophys Res Commun 305:754–760

Lappin AK, Brandt Y, Husak JF, Macedonia JM, Kemp DJ (2006) Gaping displays reveal and amplify a mechanically-based index of weapon performance. Am Nat 168:100–113

Loew ER, Fleishman LJ, Foster RG, Provencio I (2002) Visual pigments and oil droplets in diurnal lizards a comparative study of Caribbean anoles. J Exp Biol 205:927–938

López P, Martín J (2006) Lipids in the femoral gland secretions of male Schreiber's green lizards, *Lacerta schreiberi*. Z Naturforsch C 61:763–768

López P, Aragón P, Martín J (1998) Iberian rock lizards (*Lacerta monticola cyreni*) assess conspecific information using composite signals from faecal pellets. Ethology 104:809–820

Marco A (1996) Sedentarismo, áreas de campeo y selección de microhábitats en el lagarto verdinegro *Lacerta schreiberi*. Doñana Acta Vertebrata 23:45–61

Marco A (2015) Lagarto verdinegro - *Lacerta schreiberi*. In: Salvador A, Marco A (eds) Enciclopedia Virtual de los Vertebrados Españoles. Museo Nacional de Ciencias Naturales, Madrid, http://www.vertebradosibericos.org

Marco A, Pérez-Mellado V (1999) Mate guarding, intrasexual competition and mating success in males of the non-territorial lizard *Lacerta schreiberi*. Ethol Ecol Evol 11:279–286

Martín J, López P (2007) Scent may signal fighting ability in male Iberian rock lizards. Biol Lett 3:125–127

Martín J, López P (2009) Multiple color signals may reveal multiple messages in male Schreiber's green lizards, *Lacerta schreiberi*. Behav Ecol Sociobiol 63:1743–1755

Martín J, Moreira PL, López P (2007) Status-signalling chemical badges in male Iberian rock lizards. Functional Ecology 21:568–576

Maynard Smith J (1982) Evolution and the theory of games. Cambridge University Press, Cambridge

Megía-Palma R (2016) Molecular characterization of lizard parasites and their influence on colour ornaments. PhD Dissertation, Universidad Complutense de Madrid, Madrid

Moczek AP, Emlen DJ (2000) Male horn dimorphism in the scarab beetle, *Onthophagus taurus*: do alternative reproductive tactics favour alternative phenotypes? Anim Behav 59: 459-466

Montgomerie R, Hill GE, McGraw KJ (2006) Analyzing colors. In: Hill GE, McGraw KJ (eds) Bird coloration vol 1. Mechanisms and measurements. Harvard University Press, Cambridge, pp 90–147

Olsson M, Madsen T (1998) Sexual selection and sperm competition in reptiles. In: Birkhead TR, Møller AP (eds) Sperm competition and sexual selection. Academic Press, San Diego, pp 503–578

Olsson M, Shine R (1997) Advantages of multiple matings to females: a test of the infertility hypothesis using lizards. Evolution 51:1684-1688

Olsson M, Shine R, Madsen T, Gullberg A, Tegelstrom H (1996) Sperm selection by females. Nature 383:585–585

Oppliger A, Giorgi MS, Conelli A, Nembrini M, John-Alder HB (2004) Effect of testosterone on immunocompetence, parasite load, and metabolism in the common wall lizard (*Podarcis muralis*). Can J Zool 82:1713–1719

Perry G, LeVering K, Girard I, Garland T (2004) Locomotor performance and social dominance in male *Anolis cristatellus*. Anim Behav 67:37–47

Pough FH, Andrews RM, Cadle JE, Crump ML, Savitzky AH, Wells KD (2004) Herpetology, 3rd edn. Pearson, Upper Saddle River

Qvarnström A, Forsgren E (1998) Should females prefer dominant males? Trends Ecol Evol 13:498–501

R Core Team (2016) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, http://www.R-project.org/

Rohwer SA (1975) The social significance of avian winter plumage variability. Evolution 29:593-610

Rohwer SA (1982) The evolution of reliable and unreliable badges of fighting ability. Am Zool 22:531-546

Santos ESA, Scheck D, Nakagawa S (2011) Dominance and plumage traits: meta-analysis and metaregression analysis. Anim Behav 82:3–19

Sassoon DA, Gray GE, Kelley DB (1987) Androgen regulation of muscle fiber type in the sexually dimorphic larynx of *Xenopus laevis*. J Neurosci 7:3198–3206

Sinervo B, Lively CM (1996) The rock-paper-scissors game and the evolution of alternative male strategies. Nature 380:240-243

Sneddon LU, Huntingford FA, Taylor AC, Orr JF (2000) Weapon strength and competitive success in the fights of shore crabs. J Zool 250:397–403

Sokal RR, Rohlf FJ (1995) Biometry, 3rd edn. WH Freeman, New York

Stamps JA (1994) Territorial behavior: testing the assumptions. Adv Stud Behav 23:173–232

Strobl C, Boulesteix AL, Kneib T, Augustin T, Zeileis A (2008) Conditional variable importance for random forests. BMC Bioinformatics 9:307

Suleiman SA, Elamin Ali M, Zaki ZMS, El-Malik EMA, Nasr MA (1996) Lipid peroxidation and human sperm motility: protective role of vitamin E. J Androl 17:530–537

Surai PF, Kutz E, Wishart GJ, Noble RC, Speake BK (1997) The relationship between the dietary provision of α-tocopherol and the concentration of this vitamin in the semen of chicken: effects on lipid composition and susceptibility to peroxidation. J Reprod Fertil 110:47–51

Studd MV, Robertson RJ (1985) Sexual selection and variation in reproductive strategy in male Yellow Warblers (*Dendroica petechia*). Behav Ecol Sociobiol 17:101-109

Számadó S (2000) Cheating as a mixed strategy in a simple model of aggressive communication. Anim Behav 59: 221-230

Taborsky M, Oliveira RF, Brockmann HJ (2008) The evolution of alternative reproductive tactics: concepts and questions. In: Oliveira RF, Taborsky M, Brockmann HJ (eds) Alternative reproductive tactics: an integrative approach. Cambridge University Press, Cambridge, pp 1–21

Tokarz RR (1985) Body size as a factor determining dominance in staged agonistic encounters between male brown anoles (*Anolis sagrei*). Anim Behav 33:746-753

Tremellen K (2008) Oxidative stress and male infertility—a clinical perspective. Hum reprod update 14:243-258

Tuttle EM (2003) Alternative reproductive strategies in the White-throated Sparrow: behavioral and genetic evidence. Behav Ecol 14: 425-432

Vitt LJ, Cooper Jr WE (1985) The evolution of sexual dimorphism in the skink *Eumeces laticeps*: an example of sexual selection. Can J Zool 63:995–1002

Wingfield JC, Ball GF, Dufty AM, Hegner RE, Ramenofsky M (1987) Testosterone and aggression in birds. Am Sci 75:602–608

White GC, Garrott RA (1990) Analysis of wildlife radio-tracking data. Academic Press, San Diego

Chapter VI

Sexual coloration of female *Lacerta schreiberi* lizards may signal health state and potential reproductive investment

Renata Kopena, Pilar López and José Martín

Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC. José Gutiérrez Abascal 2, E-28006 Madrid, Spain

Abstract

Female animals often have conspicuous, or less conspicuous but well-recognisable by conspecifics, sexual ornaments, but their evolution and function are less-known than those of males. Female ornaments can evolve and be maintained as a result of a non-adaptive process of intersexual genetic correlation between males and females, but these traits can also indicate female quality, informing, for example, about the strength of the immune system, parasite burden and condition or potential fecundity and investment into offspring. We analyzed whether there were dichromatic differences between sexes in the lizard *Lacerta schreiberi*. We, furthermore, examined whether pigmental or structural-based coloration of females may function signalling health state or potential reproductive investment. We found that females with longer body sizes had stronger PHA-induced immune responses, females with more intense UV-blue throat coloration had less haemoparasites, and females with more intense UV-green dorsal coloration had less ticks and longer body sizes, however body size related positively with chest yellow saturation too. Relative clutch size had serious negative effects on body condition of females, but not on their immune response. Mother coloration may predict several components of reproductive investment, such as incubation time,

hatching success, and size and condition of their hatchlings. Head parameters of offspring, which can be important in survival, were strongly positively correlated with mother head sizes and negatively with the mother haemoparasite burden. Longer times spent in captivity by pregnant females resulted in lower hatching success, offspring with smaller average body size, smaller head sizes and more morphological abnormalities. Captivity time influenced negatively dorsal color saturation of females, but, surprisingly, it did not affect their body condition or immune response. These indicator signals support direct sexual selection of female ornaments. However, due to the similar basis of coloration in both gender, genetic correlation is neither excluded. Moreover, these two phenomena would increase even synergistically the fitness of individuals that prefer these color signals.

Keywords: Female sexual signals · Immune system · Parasite burden · Offspring quality · Reproductive success · Lizards

Introduction

The evolution, function and maintenance of male sexual signals are very well-researched topics, but female animals often have also conspicuous, or less conspicuous but well-recognisable by conspecifics, sexual ornaments which evolution and function are less-known than those of males (Clutton-Brock 2009; Wrigth et al 2015). In the past, female ornaments were considered only as a result of a non-adaptive process of intersexual genetic correlation due to the sexual or natural selectional effects on males' genome, which are inherited by females as well (Lande 1980). According to this, conspicuous ornaments should have negative effects on female survival and reproductive success, especially when there is maternal care, due to increased predation risk. For example, female common yellowthroat (*Geothlypis trichas*) with more vivid yellow bibs suffered more total brood loss due to predation (Freeman-Gallant et al. 2014). However, female ornaments can also evolve by direct selection. In sex-role reversed species, female ornaments can indicate competitiveness

for males (Berglund and Rosenqvist 2001). Also, females can compete for resources such as a breeding territory or a nesting hollow, or other requirements for conception or rearing of offspring (Heinsohn et al. 2005; Hegyi et al. 2008; Griggio et al. 2010). Female ornaments can indicate female quality informing about health state, strength of the immune system, parasite burden and condition (Johnsen et al. 1996; Potti and Merino 1996; Skarstein and Folstad 1996; Roulin et al. 2008; Dreiss and Roulin 2010; Kelly et al. 2012). Furthermore, these female signals can show potential fecundity and investment into offspring of the mother (Jawor et al. 2004; Siefferman and Hill 2005; Roulin et al. 2010; Potti et al. 2013; Remeš and Matysioková 2013). Genetic correlation and direct selection provide opposite predictions between female signals and fitness, but they are not mutually exclusive phenomena. Vestigial traits from genetic correlation can be the base on which can evolve ornaments by sexual selection in females. Furthermore, there could be cooperation of these two processes; choosing an attractive female partner can result benefitial for a male by producing not only showy daughters but also more attractive sons and viceversa (Amundsen 2000).

Evolution of male mate choice can be influenced by the species' breeding system, parental investment, cost of pair searching and the variance of quality among females. In case of a biased operational sex ratio (OSR), the rare sex is choosy, thus female biased OSR can result in reversed sex roles and elaborated ornaments. But at balanced OSR, when females mate with more males and "sperm mixing" exists, producing higher quantity of sperm results in higher fertilizing success for a male in sperm competition (Parker 1982; Parker et al. 1996; Parker et al. 1997). Usually males ejaculate much more sperm than necessary to fertilize all eggs. Although production of a single sperm is cheap (Dewsbury 1982), in polygynous or promiscuous mating systems, the increased requirement of sperm transfer can be costly by limited ejaculate volume (Squires et al. 1978; Savalli and Fox 1999). Male fowls (*Gallus gallus*) invest more sperm into more ornamented females, especially the good quality males (Cornwallis and Birkhead 2007). Theoretically, evolution of male mating preferences is favored and males have direct benefits if females' quality has high variance, or if the cost of mate searching is low, or if males' parental investment is high

(Burley 1977; Parker 1983; Owens and Thompson 1994; Johnstone et al. 1996; Kokko and Monaghan 2001). In most of polygynous species where females have ornaments, females access to more males and have direct benefits from multiple pair mating (Clutton-Brock 2009). When mating rate and quality variance are similar in the two sex, or both sex invest in parental care, mutual choosiness is expected (Burley 1977; Owens and Thompson 1994; Johnstone et al. 1996). The higher the parental investment of a sex is, the choosier it is, and lower quality individuals discriminate less than good quality ones (Burley 1977). However, there are some proofs that the real reason for choosiness is not parental care per se, but rather the "time-out" of sexual competition influencing potential reproductive rate (Parker and Simmons 1996). Thus, other time-consuming behavioral investments, such as mate guarding can have similar effects on choosiness.

Honesty of female signals might function through a cost as in the case of males. However, this cost can cause a trade-off between elaboration of ornaments and fecundity. Thus, sexual selection on female ornaments can be self-limited. If males choose females by their direct fecundity benefits, they may avoid females with too elaborated signals for the cost of fecundity, but if females have to invest into ornaments anyway, males will be not able to estimate potential fecundity of females. Thereby, stabilization selection can affect female sexual signals (Fitzpatrick et al. 1995). For example, carotenoid-based pelvic spine coloration of female Three-spined sticklebacks (*Gasterosteus aculeatus*) negatively relates with carotenoid content of their eggs, and males do not prefer females with redder spines (Nordeide 2002; Nordeide et al. 2006). Nevertheless, female traits can signal directly their investment into offspring. In certain species, body size or body condition of females can predict number or size of eggs and, thus, of the offspring (Itzkowitz et al. 1998; Bonduriansky 2001; Steiger 2013). Females can influence quality of their offspring by genetical and non-genetical mechanisms. Females allocate nutrients and other important biochemicals into the embryos through the placenta or into egg yolk. Egg yolk mainly consists of proteins and lipids as nutrients (Thompson and Speake 2002), plus fatty acids, antibodies, steroid hormones and antioxidants (Schwabl 1996; Wiegand 1996; Gasparini et al. 2001; Izquierdo et al. 2001; Speake et al. 2001; Boulinier and Staszewski 2008). Rapid

embryonic growth rate due to mitosis and respiration results in high oxidative levels inside of eggs (Blount et al. 2000; Surai et al. 2001a). Thus, the antioxidant content of eggs is very important for embryonic development, neonatal growth and survival of the offspring (George et al. 2001; Izquierdo et al. 2001; Johnston et al. 2007). Conspicuous color signals, such as carotenoid or pteridin-based ornaments, can signal carotenoid/pteridin content of the body, which are important antioxidants, or the content of other antioxidants (e.g. vitamin E) that allow the maintenance of the colour signals. Size or intensity of these colour patches can be related with antioxidant content of the eggs. Female Lesser black-backed gulls (*Larus fuscus*) and Zebra finches (*Taeniopygia guttata*) fed a high-carotenoid diet invest more in color intensity of ornaments and allocate more antioxidants into eggs (Blount et al. 2002; McGraw et al. 2005). Striped plateau lizards (*Sceloporus virgatus*) females have pteridin-based orange throat patches that reach peak of color intensity at around ovulation of females and this trait is preferred by males. Size of throat patches positively relates with body condition and negatively with parasite load of females, while orange chroma positively relates with body size, thus indicating phenotypic and genetical quality of the females. Furthermore, throat patch size positively correlates with antioxidant content and average mass of the eggs, and thereby, predicts a better condition and higher running speed of the offspring (Weiss 2006; Weiss et al. 2009; Weiss et al. 2011). Structural coloration also can predict female fitness. Blue tits (*Cyanistes caeruleus*) females have ultraviolet crowns, as those of males, that predict egg size and number of fledgings (Szigeti et al. 2007; Henderson et al. 2013).

We examined here the potential functions of multiple visual traits of female Schreiber's or Iberian green lizards (*Lacerta schreiberi*), a large (130 mm maximum snout-to-vent length, SVL) diurnal lacertid lizard found in the northwest, west, and central areas of the Iberian Peninsula (Marco and De la Riva 2015). For human observers, adult *L. schreiberi* lizards show a strong sexual dichromatism; males have predominantly green with small black spots backs, yellow chests and vents,and bright blue heads, while females have yellow, likely carotenoid-based, chest coloration, and white structural throat coloration. However, dorsal coloration of females is more variable, being green or brown with different

size and amount of black spots and lines. Interindividual variation in the characteristics of males' breeding coloration may signal variation in morphology, health state, dominance, and pairing status (Martín and López 2009; Kopena et al. 2014a, b), but the possible functions of female colour ornaments are unknown. The mating system of *L. schreiberi* is promiscuous, furthermore some males mate guard females to increase possibilities of paternity (Marco and Pérez-Mellado 1999). The requirement of high sperm quantity and the loss of time out of mating by mate guarding suggest the possible existence of male mate choice in this species.

In this paper, i) we measured and compared characteristics of coloration of multiple traits of males and females to test whether there were sexual dichromatic differences, ii) we investigated relationships among coloration of multiple traits, morphology and health state (immune response and parasite burden) of adult females, iii) we explored which female traits were related with offspring quality, and iv) which characteristics of colour ornaments of mothers could predict quality of their offspring.

Materials and methods

In June 2012, 28 female lizards (21 of which were gravid) and 36 males were captured in 'Valle de La Fuenfría' in the Guadarrama Mountains (40°44' N, 4°02' W; Madrid Province, Spain) and immediately transferred to "El Ventorrillo" field station of the Museo Nacional de Ciencias Naturales (Madrid province, Spain), 5 km from the capture site. Lizards were individually kept in outdoor 51×36×28 cm PVC terraria containing coconut fiber substratum and rocks for cover. Terraria were located on a half-shady forest area, which matched thermal conditions in the original habitat of lizards. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*) and house crickets (*Acheta domesticus*), dusted with calcium and vitamin powder, and water was provided ad libitum. We measured females' snout-to-vent length (SVL) to the nearest 1 mm (mean ± SE=106 ± 1 mm; range=95-116 mm). We used digital calipers to make morphological measurements (to the nearest 0.1 mm)

of the head size of females: 'head length' (mean ± SE=22.0 ± 0.2 mm; range=19.9-23.4mm), 'head height' (mean ± SE=10.5 ± 0.1 mm; range=9.8-11.5 mm) and 'jaw width'(mean ± SE=13.3 ± 0.1 mm; range=11.7-15.3 mm). Furthermore, we measured body mass of females just after egg laying (mean ± SE=24.3 ± 0.6 g; range=18.5-29.5 g) and we calculated body condition as the residuals of the lnBodyMass regression on lnSVL. Due to the different stadium of gravidity of females at capture time, one part of vitellogenesis occured in captivity, which could, potentially elicit stress responses and, thus, increase corticosterone levels in blood plasma of mothers. This process might increase corticosterone content of egg albumen, which can negatively influence development of embryo and phenotype of hatchlings. Thus, to control for the possible stress effects of captivity, we included in statistical analyses as a covariate the "captivity time" (i.e. number of days that the female spent in captivity since capture and until egg laying).

Color measurements

Male coloration was measured in the middle of May, but, due to the different stadium of gravidity of females at capture time, we decided to measure coloration in the same stadium of all females in order to be able to compare coloration. Thus, we made all measures of coloration immediately after egg laying of each individual female. We measured reflectance of lizards' coloration from 300 to 700 nm using an Ocean Optics USB2000 spectroradiometer with a DT-1000-MINI Deuterium–Halogen light source (Ocean Optics, Inc., Dunedin, FL, USA). This range of wavelengths includes the UV coloration and coincides with the spectral sensitivity of other lizard species (Fleishman et al. 1993; Loew et al. 2002). To exclude ambient light and standardize measuring distance and angle, we used a cylindrical metallic tube mounted on the fiber optic probe (Montgomerie et al. 2006). The probe was held at a 45° angle to the skin, and reflectance was always measured by the same person (PL). We measured coloration of three visually distinct large patches of coloration ("blue" throat, "yellow" chest, and "green" back of males, and "white" throat, "yellow" chest, and "brown or green" back of females) at three standardized spots: the middle of the throat (between the last chin shields and the collar; "throat"), the chest (just anterior to the

two forelimbs at the middle of the second row of scales prior to the collar; “chest”), and the back (background coloration at the middle point above the two forelimbs; “back”). At each body location, we measured coloration of three nearby points and later calculated average values for throat, chest and back coloration of each individual.

We considered separately each part of the body (throat, chest and back) and, for each part, we made one different PCA to mathematically summarize the complex reflectance spectra into three independent PCs (Endler 1990; Cuthill et al. 1999; Grill and Rush 2000; Montgomerie 2006). In these PCAs of spectral data, PC1 represents variation in intensity of coloration or brightness, and subsequent PCs represent combinations of hue and chroma (Endler 1990; Cuthill et al. 1999; Grill and Rush 2000). Moreover, the PCA allows to identify those sections of the spectrum (wavelength regions) that contribute to the observed variation, independently of their contribution to the total amount of reflectance (Montgomerie 2006). We used two sets of principal component analyses (PCAs); one set of PCAs including all animals (Table 1) to compare male and female coloration and to estimate the existence of sexual dichromatism, and other set of PCAs including only females (Table 2) to analyze relationships of coloration with female traits and mother reproductive investment.

In addition, to clarify the role of carotenoids in coloration, from the raw spectral reflectance data of each body part we calculated the carotenoid chroma [$(R_{700nm}-R_{450nm})/R_{700nm}$]. This represents relative reflectance around peak absorbance of carotenoids, thus indicating levels of carotenoid pigments incorporated into the integument (Johnsen et al. 2003; Peters et al. 2004).

Immune response

After egg laying, we measured the immune response of female lizards in vivo by using the phytohemagglutinin (PHA) injection test (Smits et al. 1999; Belliure et al. 2004; Kopena et al. 2009b). We used a pressure-sensitive spessimeter to measure thickness (to the nearest 0.01 mm) at the same point of the right hindlimb foot pad before and 24 h after injecting 0.04 mg of PHA dissolved in 0.02 ml of phosphate-buffered saline at the marked point. The

immune response was calculated as the difference between pre- and postinjection thickness measures (Smits et al. 1999; Belliure et al. 2004). No lizard showed any sign of stress or pain due to this test, and only a slight swelling of the skin, due to the immune response, which disappeared after 48 h, was appreciable. Although PHA-induced swelling is related to heightened immune cell activity, it also may involve both innate and adaptive components of the immune system (Martin et al. 2006; Salaberria et al. 2013). Thus, this test is not considered as an unambiguous index of T cell-mediated immunity per se (but see Tella et al. 2008), but rather as a multifaceted index of cutaneous immune activity. Nevertheless, we used this test here because we were interested in a standardized index of immunocompetence, reflecting health state, independent of the type of immune cells involved.

Table 1. Result and interpretation of Principal Component Analyses on different body parts of both gender.

		Eigenvalue	% of the variation	Relationship of the variables with PC scores		Interpretation
				positive	*negative*	
throat	PC1	62.87	77.62	300-310 nm	310-700 nm	Lower PC scores--> brighter throat
	PC2	13.51	16.68	300-455 nm	455-700 nm	Higher PC scores-->more saturated UV-blue throat
	PC3	2.90	3.58	335-555 nm	300-335 nm 555-700 nm	Higher PC scores-->higher relative amount of short and middle wavelengths comparing to very short and long wavelengths
	Total	79.29	97.88			
chest	PC1	57.92	71.51	-	300-700 nm	Lower PC scores-->brighter chest
	PC2	14.26	17.60	300-495 nm	495-700 nm	Lower PC scores-->more saturated yellow chest
	PC3	5.69	7.02	385-575 nm	300-385 nm 575-700 nm	Lower PC scores-->higher relative amount of UV and yellow wavelengths comparing to blue and green wavelengths
	Total	77.87	96.13			
back	PC1	61.22	75.58	-	300-700 nm	Lower PC scores-->brighter back
	PC2	13.93	17.20	300-455 nm	455-700 nm	Lower PC scores-->more saturated green or brown back
	PC3	3.09	3.82	300-340 nm 495-620 nm	340-495 nm 620-700 nm	Higher PC scores--> higher relative amount of very short UV and green-yellow wavelengths comparing to long UV-blue and long wavelengths
	Total	78.25	96.60			

Table 2. Result and interpretation of Principal Component Analyses of female coloration on different body parts.

		Eigenvalue	% of the variation	Relationship of the variables with PC scores *positive*	*negative*	Interpretation
throat	PC1	60.04	74.12	-	300-700 nm	Lower PC scores--> brighter throat
	PC2	12.61	15.56	300-470 nm	470-700 nm	Higher PC scores-->more saturated UV-blue throat
	PC3	4.38	5.41	375-605nm	300-375 nm 605-700 nm	Higher PC scores-->higher relative amount of short-middle wavelengths comparing to very short and long wavelengths
	Total	77.02	95.09			
chest	PC1	50.40	62.22	-	300-700 nm	Lower PC scores-->brighter chest
	PC2	16.78	20.71	300-490 nm	490-700 nm	Lower PC scores-->more saturated yellow chest
	PC3	9.84	12.15	385-590 nm	300-385 nm 590-700 nm	Lower PCscores-->higher relative amount of very short and long wavelengths comparing to short-middle wavelengths
	Total	77.02	95.08			
back	PC1	55.78	68.86	-	300-700 nm	Lower PC scores-->brighter back
	PC2	16.73	20.66	300-475 nm	475-700 nm	Lower PC scores-->more saturated green or brown back
	PC3	4.79	5.91	335-425 nm 485-610 nm	300-335 nm 425-485 nm 610-700 nm	Higher PC scores--> higher relative amounts of short and green-yellow wavelengths comparing to very short UV, blue and very long wavelengths
	Total	77.30	95.43			

Parasite burden

We counted the number of ectoparasites (*Ixodes* sp., Ixodidae) fixed on each individual female at capture time (N=28, tick prevalence=79%, ectoparasite median intensity=3.5, $CI_{95\%}$=3.4-8.7, Range=1-20). Furthermore, we counted haemoparasites by using blood smears made after egg laying. A smear was made on a microscope slide from blood taken from a large subcutaneous vessel on the ventral side of the body by using a 25-G insulin injection needle and a 9-µl heparinized hematocrit tube. Blood smears were air-dried, fixed in absolute methanol for 10 min, and then stained in Giemsa diluted 1:9 with phosphate buffer (pH 7.2) before their examination for parasites. On mounted slides, half a smear, chosen at random, was scanned entirely at 200x along the length of the slide, looking for extraerythrocytic protozoa (Merino and Potti 1995). Numbers of intraerythrocytic

parasites were estimated at 400x by counting the number of parasites per 2,000 erythrocytes. The only haemoparasites we found were haemogregarines. Haemoparasite prevalence was estimated as the percentage of infected lizards (prevalence=79%). Parasite median intensity was estimated for each infected host as the number of infected red blood cells found in approximately 2,000 cells (median=25.5, $CI_{95\%}$=20.0-53.2, Range=1-142). Because values of tick and haemogregarine infection were not normally distributed, we used square-root transformation to achieve normality (Kolmogorov–Smirnov test for both variables: d=0.11, p>0.20) and we used the transformed variable in further analyses.

Eggs and hatchling husbandry

Females laid 281 eggs in their own containers (between 27 June and 24 July) and eggs were immediately transferred as clutches to closed plastic boxes (13x13 cm^2) filled with moistened perlite (1 g perlite:1 g water). Plastic boxes with eggs were placed in an incubator at 27.5 °C (IRE-160; 94x60x60 cm; Raypa). When incubation of eggs was closed to end, they were monitored every two hours between 0800-2200h searching for newborns. Immediately after hatching (between 20th August and 13th September), the 181 born offspring (hatching success=64.4 %) were measured with digital calipers (to the nearest 0.1 mm) (snout-to-vent length (SVL), mean ± SE=33.5± 0.1 mm; range=29.6-36.5 mm; 'head length', mean ± SE=9.1± 0.1 mm; range=8.4-9.7 mm: 'head height', mean ± SE=4.5± 0.1 mm; range=3.8-5.0 mm; 'jaw width', mean ± SE=5.2± 0.1 mm; range=4.7-5.7 mm) and their 'body mass' determined with a digital scale (to the nearest 0.01 g)(mean ± SE=0.95± 0.01 g; range=0.66-1.23 g). Body condition was estimated using the residuals of the regression of log-body mass on log-SVL at hatching. We observed in some of the offspring morphological abnormalities of feet and tail. The absence/presence of undeveloped or curved tail and of supernumerary toes in each of the four feet were noted to made an index of "abnormality degree" (potentially ranging from 0 to 5 depending on the number of abnormalities observed) (mean ± SE=0.36± 0.05; range:0-3). After measurement, offspring were placed in outdoor 51×36×28 cm PVC terraria in the same conditions as females for further observations.

Data analyses

Coloration characteristics (PC scores) of females and males were compared with ANOVAs separately for each PCs. To estimate female quality and cost of vitellogenesis, we used backward stepwise general regression models (GRM) in STATISTICA Software (StatSoft, Inc, Tulsa, OK, USA) to analyze whether body condition after egg laying (dependent variable) depended on female size, parasite burden, captivity time and relative clutch size (independent variables). We also used a stepwise GRM to test whether immune response after egg laying (dependent variable) was affected by female size, parasite burden, condition, captivity time and clutch size (independent variables). Furthermore, we analyzed the effects of each of these female quality traits (dependent variables), on coloration using a backward stepwise GRM with PC scores describing coloration as independent variables.

We also used backward stepwise GRMs to investigate effects of female quality traits (SVL*, head length*, head width*, head height*, condition after egg laying, tick and haemoparasite burden, captivity time) and female coloration (PC scores) as independent variables on each of the offspring traits (SVL, head length, head width, head height, body condition, abnormality degree, incubation time, clutch size; the average values of all the offspring of each female) (dependent variables). Independent variables of females signed by asterisk above were matched in the GRMs with dependent variables showing the same trait of offspring (e.g., mother SVL was only included in the model where the dependent variable was offspring SVL).

Because female size may affect clutch size, we calculated relative clutch size of each female using the residuals of the regression of clutch size on female SVL. Furthermore, since laying date and incubation time of eggs and traits of offspring may be strongly influenced by clutch size, we used clutch size adjusted variables as dependent variables.

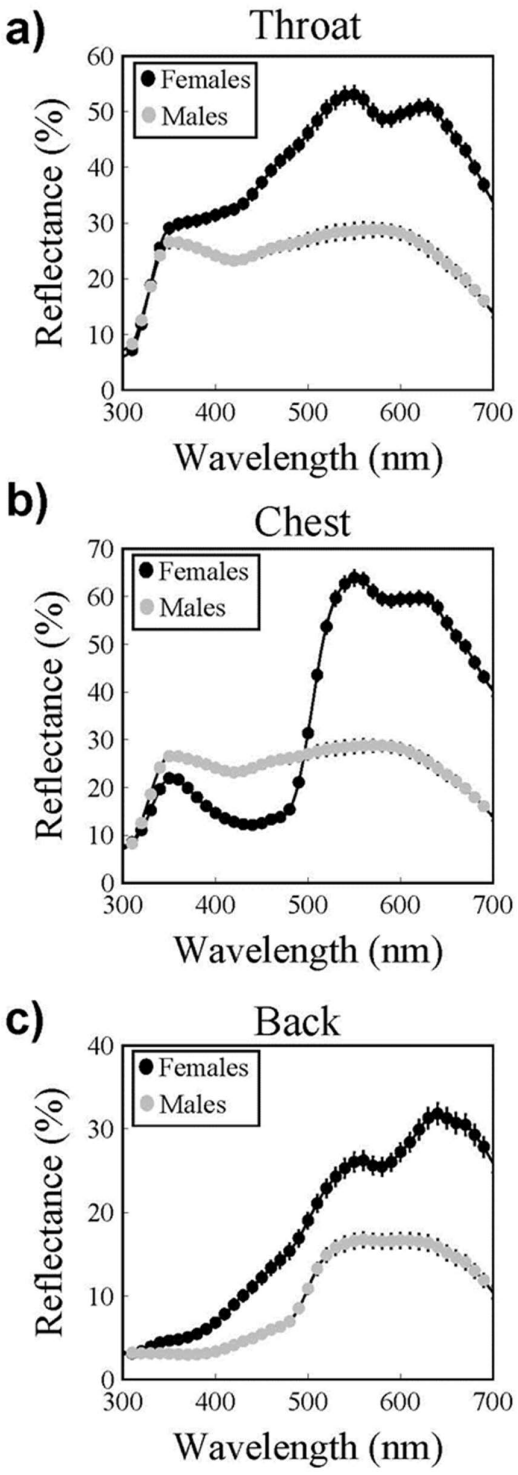

Fig. 1 Mean (± SE showed at 5nm intervals) reflectance spectra of throat (**a**), chest (**b**) and back (**c**) of females (black dots) and males (grey dots).

Results

Intersexual differences in coloration

In comparison with males, females had significantly lower values of throat PC1 ($F_{1,60}$=110.26, p<0.001), chest PC1 ($F_{1,60}$=56.07, p<0.001) and back PC1 coloration ($F_{1,60}$=78.27, p<0.001), which mean that the three measured body parts of females were brighter than those of males. Furthermore, females had chest coloration with lower chest PC2 values than males ($F_{1,60}$=7.58, p=0.008), so female chest was more yellowish. This is likely explained because the female chest contents more carotenoids than the male chest, which was supported by the higher chest carotenoid chroma of females (males: 1.27 $\pm$ 0.01; females: 1.34 $\pm$ 0.01; $F_{1,60}$=22.67, p<0.001). Males had significantly higher values of throat PC2 ($F_{1,60}$=7.93, p=0.007) and back PC3 ($F_{1,60}$=6.05, p=0.017) than females, which means that the throat of males had more intense UV and more saturated blue coloration. Furthermore, the back of males had more intense very short UV wavelengths (300-335 nm) and was greener. However, males and females did not significantly differ in throat PC3 ($F_{1,60}$=0.47, p=0.50), chest PC3 ($F_{1,60}$=2.88, p=0.095) and back PC2 ($F_{1,60}$=1.64, p=0.205).

Condition and immune response of females after egg laying

Mother condition after laying was negatively and significantly related with relative clutch size only (stepwise GRM, model: R^2=0.30, $F_{1,18}$=9.05, p=0.008; relative clutch size: β=-0.58, t=-3.01, p=0.008). Thus, females that had higher relative clutch sizes had a lower body condition.

Immune response of females after egg laying was positively and significantly correlated with SVL (stepwise GRM, model: R^2=0.31, $F_{1,17}$=8.90, p=0.008; SVL: β=0.59, t=2.98, p=0.008), but there were no significant relationships with parasite burden, condition, captivity time or relative clutch size. Since PHA-induced immune response was size-dependent, we calculated relative immune response using the residuals of the regression of immune response on SVL and we used it in subsequent analyses.

Relationships of coloration with morphology and health state of females

Females with higher values of throat PC2 (i.e., more intensive UV and blue throat coloration) had significantly lower haemoparasite loads (stepwise GRM, model: R^2=0.24, $F_{1,17}$=6.82, p=0.018; haemoparasites: β=-0.54, t=-2.61, p=0.018). Females with lower values of chest PC2 (i.e., more saturated yellowish chest coloration) had significantly longer body sizes (stepwise GRM: R^2=0.29, $F_{1,17}$=8.50, p=0.01; SVL: β=-0.58, t=-2.92, p=0.01).

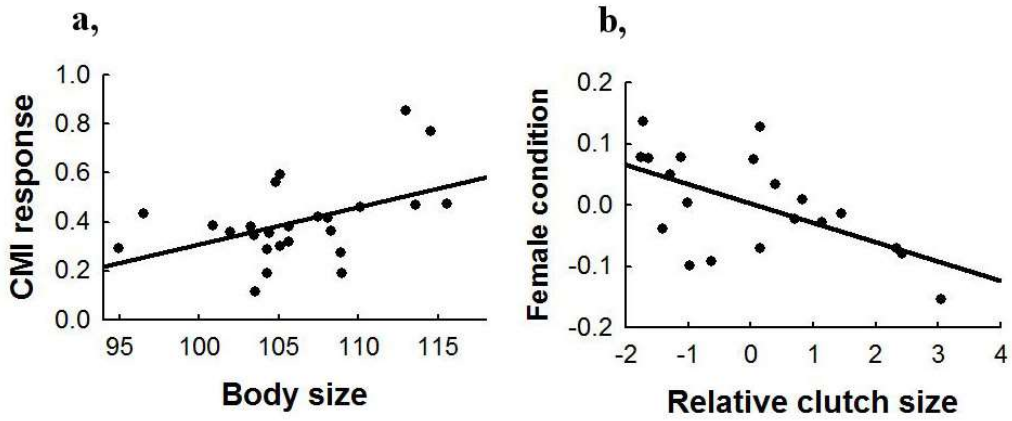

Fig. 2 Relationships between female body size and PHA-induced immune response (**a**) and between relative clutch size and condition after egg laying (**b**).

Back PC2 values of female back coloration were significantly related with captivity time only (stepwise GRM, model: R^2=0.21, $F_{1,17}$=5.69, p=0.029; captivity time: β=0.50, t=2.39, p=0.029). Thus, the longer the time females were in captivity, the lower the saturation of both back brown and green coloration was. Back PC3 was significantly related with body length and number of ticks (stepwise GRM, model: R^2=0.47, $F_{2,16}$=8.88, p=0.003; SVL: β=0.56, t=3.26, p=0.005; tick load: β=-0.44, t=-2.58, p=0.02). Thus, females with greener and more intensive UV back coloration had less ectoparasites and longer body sizes. The rest of coloration traits (PC scores) were not significantly correlated with any variable describing morphology or health status (i.e., no variable was included in the final models).

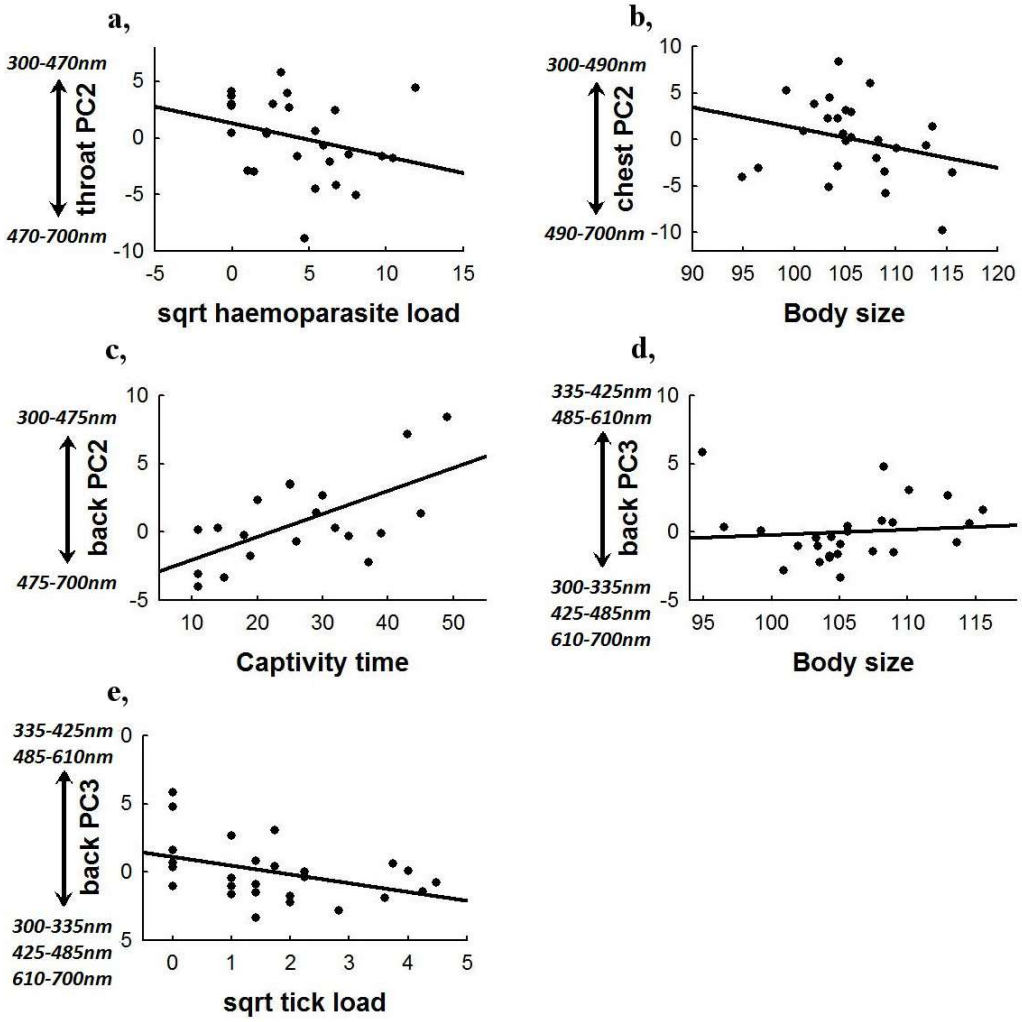

Fig. 3 Relationship between haemoparasite burden and throat PC2 (**a**), between body size and chest PC2 (**b**), between captivity time and back PC2 (**c**), between body size and back PC3 (**d**) and between tick load and back PC3 (**e**) of females.

Relationships of offspring traits with mother quality traits

Relative clutch size was not influenced by parasite load, nor by captivity time. However, hatchling number (stepwise GRM, model: R^2=0.39, $F_{1,19}$=13.94, p=0.001; captivity time: β=-0.65, t=-3.73, p=0.001) and hatching success (stepwise GRM, model: R^2=0.43, $F_{1,19}$=16.38, p=0.0007; captivity time: β=-0.68, t=-4.05, p=0.0007) were only affected significantly by captivity time. Thus, captivity stress did not affect the number of eggs but had significant negative effects on viability of eggs. Incubation time and egg laying date were no significantly related with mother traits.

Offspring SVL (stepwise GRM, model: R^2=0.29, $F_{1,16}$=7.95, p=0.012; captivity time: β=-0.58, t=-2.82, p=0.012) and head length (stepwise GRM, model: R^2=0.62,

$F_{1,16}$=28.63, p<0.0001; captivity time: β=-0.80, t=-5.35, p<0.0001) were influenced negatively by captivity time, whereas offspring average abnormality degree was positively related with captivity time (stepwise GRM, model: R^2=0.41, $F_{1,16}$=12.76, p=0.0025; captivity time: β=0.67, t=3.57, p=0.0025). Thus, captivity stress seemed to result in hatchlings with smaller body sizes, shorter heads and more morhological abnormalities.

Head height of hatchlings was significantly and positively related with mother head height and significantly and negatively related with haemoparasite load of their mothers (stepwise GRM, model: R^2=0.26, $F_{2,15}$=3.96, p=0.042; HH: β=0.53, t=2.41, p=0.029; haemoparasite load: β=-0.48, t=-2.16, p=0.048). Thus, mothers with higher heads had hatchlings with higher heads, but haemoparasite load of mothers had negative effects on hatchling head height. Also, head width of hatchlings was related positively with mother head width, while it was related negatively with haemoparasite load and days in captivity (stepwise GRM, model: R^2=0.51, $F_{3,14}$=6.86, p=0.004; HW: β=0.83, t=4.01, p=0.001; haemoparasite load: β=-0.53, t=-2.61, p=0.02; captivity time: β=-0.53, t=-2.99, p=0.01). Thus, mothers with wider heads had hatchlings with wider heads, but haemoparasite load of mothers and captivity stress had negative effects on hatchling head width. Female quality traits did not significantly affect average condition of their hatchlings.

Relationships of offspring traits with mother coloration

Absolute and relative clutch size were not significantly related with any female color trait, but both hatchling number (stepwise GRM, model: R^2=0.24, $F_{1,18}$=6.84, p=0.017; back PC2: β=-0.52, t=-2.62, p=0.017) and hatching success (stepwise GRM, model: R^2=0.17, $F_{1,18}$=5.02, p=0.038; back PC2: β=-0.47, t=-2.24, p=0.038) were negatively and significantly related with back PC2 coloration of females, which means that females with higher values of medium and long wavelength (475-700 nm) back coloration had more offspring, both considering absolute values or relative to clutch size (i.e., higher hatching success). Egg laying date (adjusted for clutch size) was significantly and positively related with back PC2 coloration of females (stepwise GRM, model: R^2=0.33, $F_{1,18}$=10.38, p=0.0047; back PC2: β=0.60, t=3.22, p=0.0047). Thus, females with higher values of medium and long wave-

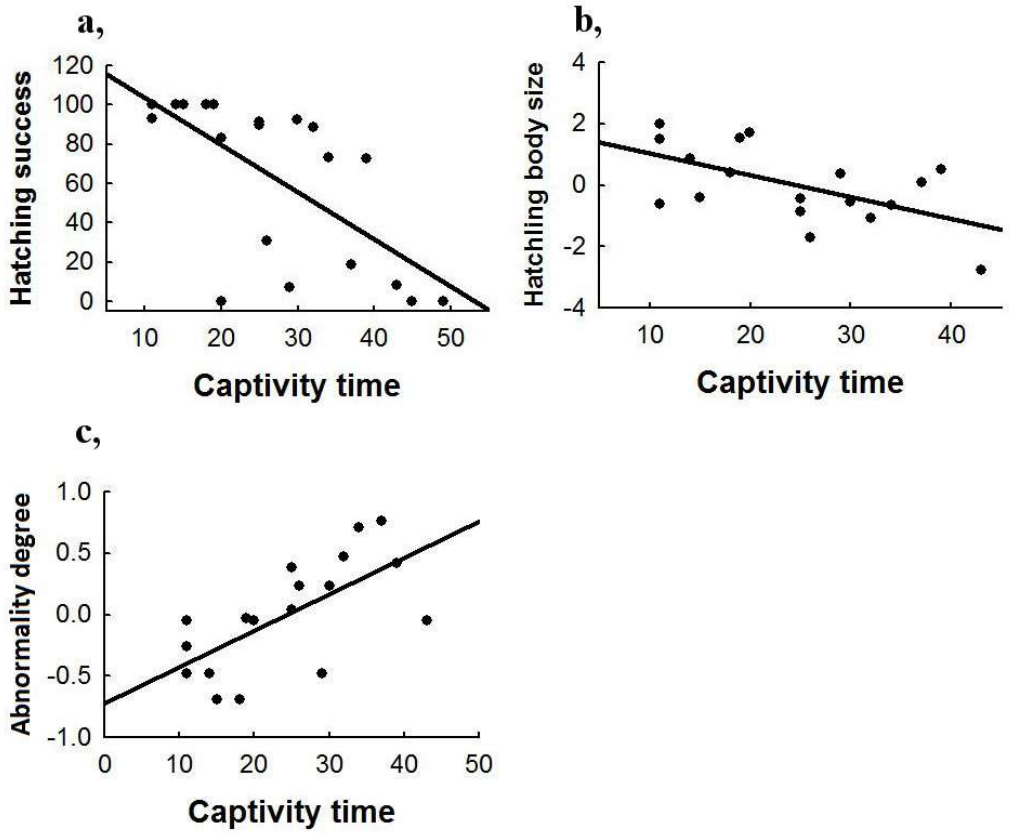

Fig. 4 Relationship between captivity time and hatching success (**a**), hatchling body size (**b**), abnormality degree (**c**).

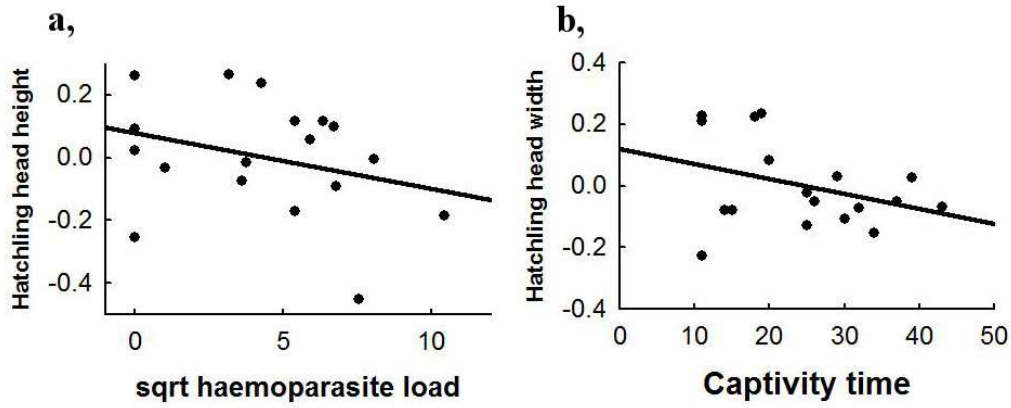

Fig. 5 Relationship between haemoparasite load and head height of hatchlings (**a**) and between captivity time and head width of hatchlings (**b**).

lengths (475-700 nm) in back coloration laid their eggs earlier. Incubation time (adjusted for clutch size) was significantly and negatively related with the mother's chest PC2 and back

PC3 coloration values (stepwise GRM, model: R^2=0.46, $F_{2,15}$=8.25, p=0.004; chest PC2: β=-0.52, t=-2.79, p=0.014; back PC3: β=-0.68, t=-3.63, p=0.002). Thus, eggs from females with more yellowish chests and less intense UV and green back coloration had longer incubation times.

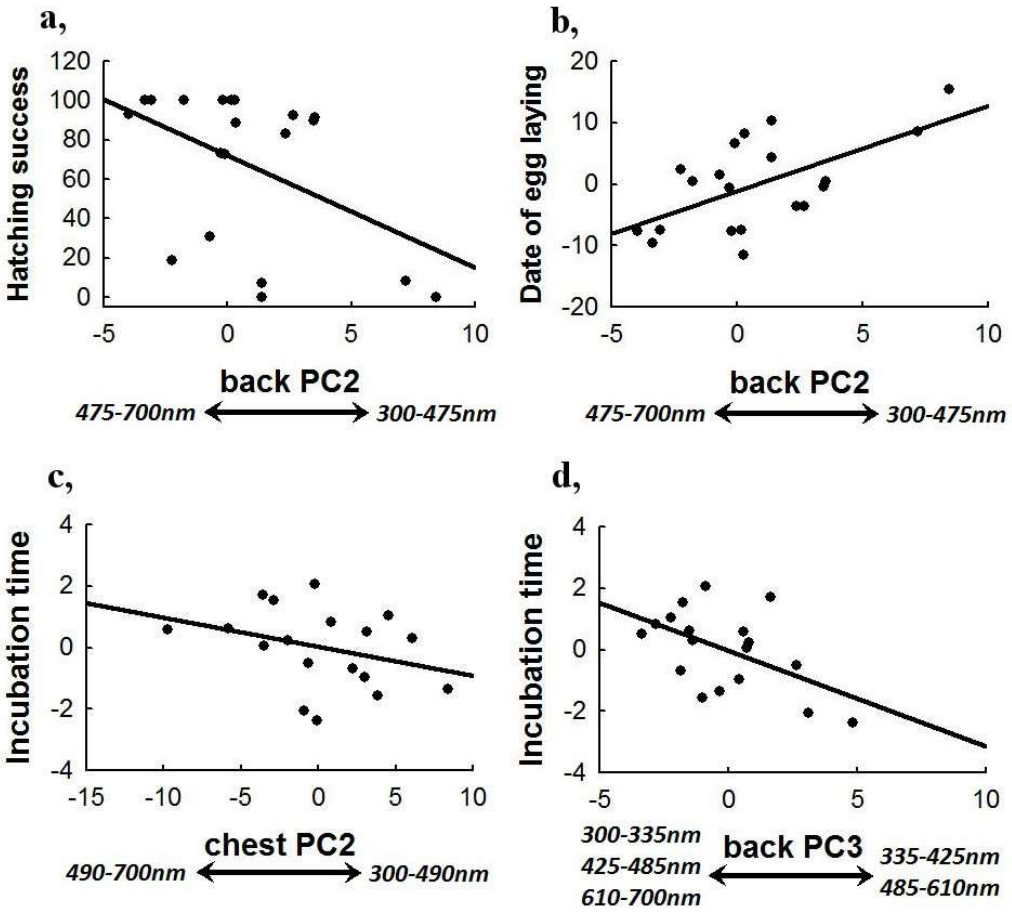

Fig. 6 Relationship between back PC2 of females and hatching success (**a**) and date of egg laying (**b**), and chest PC2 (**c**) and back PC3 of females (**d**) relationship with incubation time.

Average offspring SVL (adjusted for clutch size) was significantly and negatively related with throat PC3 and back PC2 coloration values of their mothers, while offspring SVL was significantly and positively correlated with back PC1 coloration (stepwise GRM, model: R^2=0.63, $F_{3,14}$=10.36, p=0.0007; throat PC3: β=-0.47, t=-3.09, p=0.008; back PC1: β=0.43, t=2.84, p=0.013, back PC2: β=-0.46, t=-3.02, p=0.009). Thus, females with higher values of very short (300-375 nm) and very long (605-700 nm) wavelength throat coloration

and duller but higher values of medium and long wavelengths (475-700 nm) in back coloration had on average larger hatchlings. Hatchling head length was significantly and negatively correlated with back PC2 coloration of their mothers (stepwise GRM, model: R^2=0.25, $F_{1,16}$=6.54, p=0.021; back PC2: β=-0.54, t=-2.56, p=0.021), while hatchling head width was significantly and negatively related with throat PC3 coloration of their mothers (stepwise GRM, model: R^2=0.20, $F_{1,16}$=5.35, p=0.034; throat PC3: β=-0.50, t=-2.31, p=0.034). Thus, females with less saturated back coloration (i.e., lower values of 475-700 nm range) had hatchlings with longer heads, and females with higher UV and long wavelength intensity in throat coloration (i.e., higher values of 300-375 nm and 605-700 nm) had hatchlings with wider heads. Head height of hatchlings was not related to any color trait of their mothers.

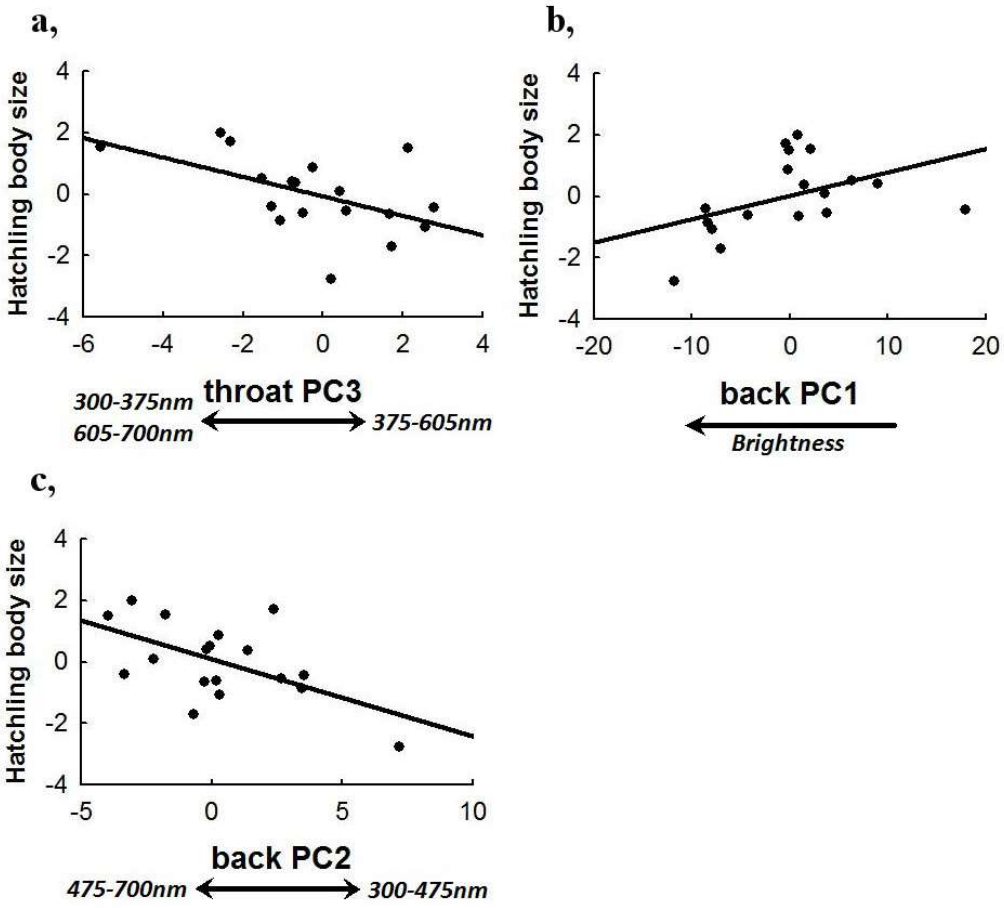

Fig.7 Relationship between body size of hatchlings and throat PC3 (**a**), back PC1 (**b**) and back PC2 of females (**c**).

Body condition of hatchlings was significantly and positively related with throat PC1, throat PC2 and chest PC1, and significantly and negatively related with throat PC3 and chest PC2 (stepwise GRM, model: R^2=0.72, $F_{5,12}$=9.92, p=0.0006; throat PC1: β=0.55, t=4.10, p=0.001, throat PC2: β=0.31, t=2.42, p=0.032, throat PC3: β=-0.49, t=-3.48, p=0.0046, chest PC1: β=0.57, t=4.28, p=0.001, chest PC2: β=-0.33, t=-2.54, p=0.026). Thus, mothers with duller and more intensive UV and bluish throats, and duller but more yellowish chests had offspring with a better condition. Average abnormality degree of hatchlings was not significantly related to any color trait of their mothers.

Discussion

Our results strongly suggest that the dichromatic sexual coloration of female *L. schreiberi* lizards may signal health condition and may also reliably predict quality of their offspring.

Comparing coloration of males and females, we found that males have more saturated UV and blue throats and greener backs. The base of throat coloration is likely based on the arrangement of iridophores and their reflective platelets and the amount of melanin in the skin in both males and females. However, males likely can produce more melanin and more regularly arranged iridophores and reflective platelets, resulting in more intensive UV and blue throat coloration. All adult males have green back coloration, but, in this population, only ca. 25% of females have green coloration on the back, while most of females have brown backs. Both brown and green back coloration can result from the combined effect of melanin, iridophores and carotenoids. However, quantity and placement of the chromatophores are different (Kuriyama et al. 2006; Saenko et al. 2013). Females are brighter on all body parts and have more yellowish chests with higher carotenoid chroma than males. Coloration can be costly for both sex, and with their promiscuous mating system, this can suggest the existence of mutual mate choice. Furthermore, due to the similar based coloration of each body part in both males and females, genetic correlation can be the reason

of the existence of this coloration in females. Whereas, due to the honesty of the characteristic of these visual signals, female coloration traits may function as indicator signals. These two phenomena can exist together synergistically increasing fitness of individuals that prefer these color signals.

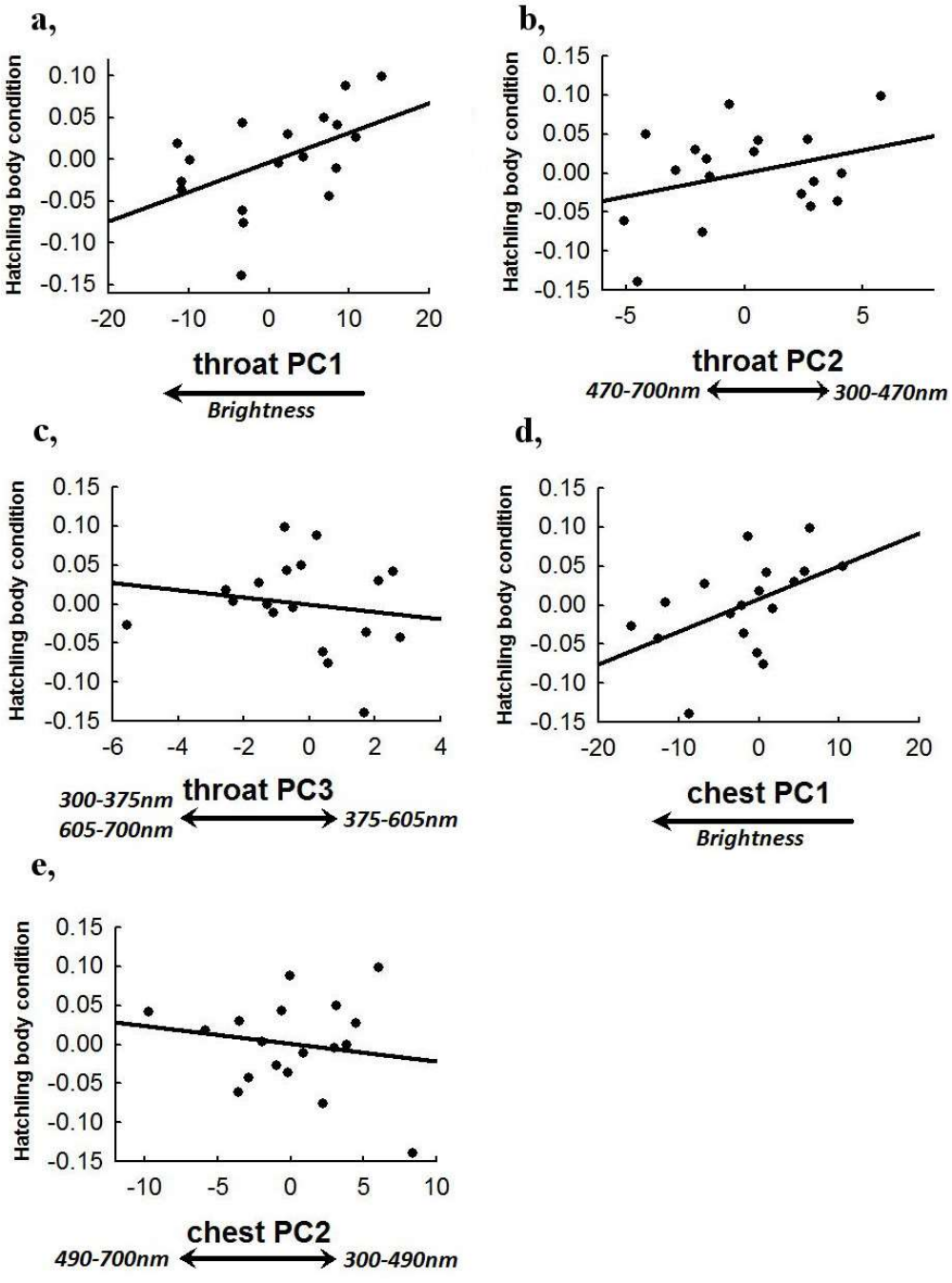

Fig. 8 Relationship of throat PC1 (**a**), throat PC2 (**b**), throat PC3 (**c**), chest PC1 (**d**), chest PC2 (**e**) of females with body condition of hatchlings.

Costs of vitellogenesis seemed to have serious effects on condition of females, but not on their immune responses. Mother condition after egg laying was lower when relative clutch size had been higher. The current actual reproductive investment can increase current reproductive success, but it can decrease the future reproductive success, such as occurs in *Lacerta vivipara* lizards (Bleu et al. 2011). The immune response of females was positively related with their body size, which is a frequent phenomenon (see e.g., Tobler et al. 2011; Vinkler et al. 2012), which might be caused by larger animals having larger cellular reserves and larger volume of skin prone to inflammation (Vinkler et al. 2012).

Coloration of mothers may signal health condition, at least after egg laying. Females with more intense UV and blue throat coloration had less haemoparasites, which can be caused by the positive effect of antioxidants on either the placement of reflective platelets in iridophores or the production of melanin. Haemoparasites can decrease antioxidant level of the body in a complex way: (i) blood parasites can elicit physiological stress reaction (Tomás et al. 2005), (ii) increase lipid peroxidation in the blood (Commins et al. 1998, Deger et al. 2009, Saleh 2009), (iii) acut phase of immune reaction can decrease carrier molecules of lipid soluble antioxidants (Das et al. 1996). Bigger females had more saturated carotenoid-dependent yellow chests. This phenomenon is not rare, for example, common yellow-throat (*Geothlypis trichas*) females with bigger and more saturated carotenoid signals are bigger and older (Freeman-Gallant et al. 2014). In northern cardinals (*Cardinalis cardinalis*), red-orange bill color and redness of underwing feathers of female correlate with body size (Jawor et al. 2004). Bigger (and likely older) females may increase carotenoid intake by their longer life experience. Furthermore, this can contribute to develop stronger immune responses in bigger females, because carotenoids may have important roles in the immune system (Chew and Park 2004). However, carotenoid supplementation did not increase PHA-induced immune response of males in *L. schreiberi* (Kopena et al. 2014b). Also, mothers with more intense UV and green dorsal coloration had less ticks and longer body sizes (and, thereby, stronger immune responses). Maybe females with green dorsal coloration had better carotenoid absorbing intestinal system and allocation into the skin, but if females had more carotenoids, they would have a more effective immune system and a higher parasite

resistance. Signalling of parasite burden by coloration is very frequent in females of other animals; female pied flycatchers (*Ficedula hypoleuca*) with forehead white patches have less *Trypanosoma* burden (Potti and Merino 1996). In arctic charrs (*Salvelinus alpinus*), brighter red females have less parasites (Skarstein and Folstad 1996). *Sceloporus virgatus* female lizards with smaller orange throat patches have higher parasite burden (Weiss 2006). Female terrapins (*Mauremys leprosa*) with brighter limb stripes have a better immune response (Ibáñez et al. 2013).

Number of eggs laid by female *L. schreiberi* was determined only by mother size, but parasite burden did not affect absolute or relative clutch size. Similarly, we did not found effects of parasites on number of hatched offspring, nor on hatching success. Also, date of egg laying and incubation time of eggs were not influenced by parasite burden, nor by condition of mothers.

Coloration of female *L. schreiberi* did not predict their relative clutch size, but females with more saturated back coloration had more hatchlings and a higher hatching success. Furthermore, more saturated back colored females laid their eggs earlier, which provide longer time for the offspring to increase their condition before hibernation. The chromatophoral background that explain the higher saturation (higher back PC2: less reflection between 300-475 nm but more between 475-700 nm) in green and brown coloration is less known. A higher production of melanin, absorbing more light, together with a higher background reflectance, increasing long wavelengths, may result in a higher saturation of green and brown dorsal coloration. Melanin production can be influenced by protein or calcium consumption (reviewed in McGraw 2008), which can also increase egg development inside of the mother body. Other possibility is that females producing more melanin can increase body temperature faster (Cloudsley-Thompson 1999; Reguera et al. 2014). Fast body warming can increase metabolism, maybe resulting in a higher rate of egg development. Furthermore, females with more melanin would need shorter basking times to attain an appropriate body temperature, thus, decreasing predation risk. However, a higher background reflectance can decrease the warming effect. Furthermore, similar saturation effects may occur with only a more precise arrangement of reflecting platelets. Specially

organized, appropriate thick, platelets can reflect appropriate long wavelengths, and the more regular the arrangement of platelets is, the higher the reflectance is at middle (green) or long (brown) wavelengths and the lower the reflectance is at shorter wavelengths. Structural coloration can be influenced by environmental stress (Kemp and Rutowski 2007; Mäthger and Hanlon 2007). Less environmental stress or a better stress-tolerance (for example by having higher levels of antioxidants) can induce more intense structural coloration. Arrangement of platelets in iridophores can be regulated by the endocrine and nervous systems, both can induce contractile activity of the actin filaments, changing space of purine crystals. However, results are inconsistent in different species (Ligon and McCartney 2016). Furthermore, vitamin A_1 is related with ontogenetic changes in fish chromatophores (Miwa and Yamano 1999). Thus, health state and carotenoid supply transforming vitamin A_1 may influence both saturation of dorsal coloration and investment into reproductive success in female *L. schreiberi*. In northern cardinal (*Cardinalis cardinalis*), more colorful females have earlier clutches and more fledglings (Jawor et al. 2004). Female pied flycatcher (*Ficedula hypoleuca*) with bigger white wing patches breed earlier and have more hatchlings, while the presence of a white forehead patch predicts more fledglings (Morales et al. 2007).

Eggs from female *L. schreiberi* with more yellowish chests had longer incubation times, however, greener backed females had a bit shorter incubation time of eggs. However, incubation time can be prolonged by strong temperature fluctuations (Smits et al. 1999) leading to lower hatching success (Warner and Shine 2009). In our case, longer incubation may provide more time for the normal development of the embryo at stable temperatures. Due to fast metabolism, production rate of free radicals is very high inside of the eggs, and, thus, embryos require large amounts of antioxidants for their normal development (Blount et al. 2000; Surai et al. 2001a). Antioxidant capacity of carotenoids is higher in lower oxygen pressure, as it occurs inside of the eggs (Surai et al. 2001b). Thus, investment of carotenoids and other antioxidants into yolk can increase survival of the eggs and offspring quality. Furthermore, beside the amount of nutrition, longer incubation times can provide enough time for appropriate development of the embryo inside the egg, in a safe place. In *Sceloporus*

virgatus female lizards, pterin-based orange throat patch size and chroma predict average egg mass and more carotenoids and antioxidants in the eggs (Weiss 2006; Weiss et al. 2011). Carotenoid-rich *L. schreiberi* females might invest more antioxidants into eggs, resulting in longer incubation times. However, greener backed females had eggs with shorter incubation times, but because this coloration is likely genetically determined, it probably did not depend on whether females can afford to express it and this process may deprive some carotenoids from eggs. However, we did not find negative effects of a shorter incubation time of females with greener dorsal coloration on offspring quality, and this idea contradicts why these females had less ectoparasites. Other possible reason is that these embryos have faster developing rate. In a field study, we recaptured some females and found that green back colored adult females have a bit faster growing rate (N=3; increasing per year: 10 mm) than brown back females (N=4, 7mm). However, sample size was very small. Furthermore, a positive relation between body size and more intensive UV-green dorsal coloration may support the hypothesis of faster growing of green females. But we also found that males diet supplemented with carotenoids had a higher body conditions, which may increase their growth rate (Kopena et al. 2014b). Maybe this genetic or nutrition based higher growing rate can be very beneficial for embryo by allowing earlier hatching and more time for feeding and reach a better condition before the first hibernation period. This is because body size and condition can be the strongest selective factors for winter survival of hatchling lizards (Civantos et al. 1999; Civantos and Forsman 2000), partly, because the hyperglycemic cryoprotective system can be the base of winter survival in *L. schreiberi*, such as it occurs in *L. vivipara* (Costanzo et al. 1995; Grenot et al. 2000).

Head height and width of offspring were strongly influenced by the mother's head size and haemoparasite burden. Higher and wider headed offspring can eat bigger insects, increasing growth rates. Thus, the mothers', possibly heritable, head size may positively affect offspring development. In contrast, the mother haemoparasite burden may affect negatively to growth rates of hatchlings, thereby, influencing negatively the survival probability, competitive ability and, maybe, mature time of offspring. Thus, it would be

worth for males to recognize and prefer mating with parasite resistant females, because this can increase their future fitness.

Interestingly, parasites did not effect on clutch size or hatchling number, only head size of hatchlings. Since resources of mothers are limited, trade-off can evolve between quantity and quality of hatchlings (Roff 1992, Stearns 1992, Schwarzkopf et al. 1999, Caley et al. 2001). This trade-off can depend on condition of both mother and environment, and not always observable, furthermore, it can change by years (Sinervo 1990, Schwarzkopf 1992). Haemoparasites may decrease allocation of antioxidants and fat (Schall 1983) into the egg yolk, furthermore, hemogregarines produce cysts and meronts in the liver that may disturb process of vitellogenesis (Telford 2008, Roca and Galdón 2010). Females may apply this trade-off according to environmental condition to maximize their reproductive success. In our case, on an area that is rich in small sized food resources, it is worth to allocate more, but smaller headed hatchlings because their survival probabilities are enough high with appropriate food.

Mothers with higher UV throat intensity and more saturated but - maybe because of more melanin - less bright dorsal coloration had average bigger hatchlings. Mothers with more saturated dorsal coloration also had offspring with longer heads, and females with more intensive UV throat coloration had hatchlings with wider heads. However, these relationships might result from simple body size correlations. Mothers that express throats with higher UV intensity require more antioxidants because developmental stress can influence structural coloration (Kemp and Rutowski 2007; Mäthger and Hanlon 2007). Furthermore, carotenoids and vitamin E can increase structural-based throat brightness of male *L. schreiberi* (Kopena et al. 2014a). Thus, these females might invest more antioxidants into egg yolk to promote offspring size (George et al. 2001; Biard et al. 2005). Furthermore, a more melanized back of females may increase offspring development in eggs not only because of the date of egg laying or hatching success, but because it can increase hatchling size (see above possible reasons for a better development).

More saturated but less bright UV-blue throats and more saturated but less bright chests of mothers can predict hatchlings with a better condition. Structural coloration also

can indicate a better investment in reproduction, such as in blue tits, where females with more intensive UV crown have bigger egg size (Szigeti et al. 2007) and more fledglings (Henderson et al. 2013). Furthermore, lower UV-blue throated *L. schreiberi* females have more haemoparasites, and diseases can decrease carotenoid investment into egg yolk in birds (Surai et al. 2001b). Furthermore, these females may have more antioxidants to be able to afford both a more intensive structural throat coloration and a chest with more carotenoids. *Sceloporus virgatus* female lizards with bigger orange throat patches have more antioxidants in egg yolk and produce offspring with better condition (Weiss et al. 2009; Weiss et al. 2011). Carotenoid-treated females of blue tits have bigger offspring with better immune systems and that develop more yellow plumages (Biard et al. 2005).

Stress induced by the presence of predators can increase plasma corticosterone in birds (Silverin 1998; Scheuerlein et al. 2001). Increased maternal corticosterone increases egg albumen corticosterone content (Hayward and Wingfield 2004; Rubolini et al. 2005), which can cause higher embryonic mortality, developmental instability of skeletal traits, impaired development, lower hatching body mass and slower growing of hatchlings (Mashaly 1991; Heiblum et al. 2001; Eriksen et al. 2003). We found, that a longer captivity time elicited a lower lower hatching success and a lower number of hatchlings, which were of smaller average body size, with smaller head length and width at hatching, and, furthermore, with more morphological abnormalities (i.e., supernumerary toes on hind limbs and tail deformation). Captivity time negatively influenced back color saturation of females, likely by decreasing melanin content and iridophore reflectance of the skin. Decreasing melanin content of the skin can be of nutrition origin (reviewed in McGraw 2008). Maybe mealworms and crickets, used as food in captivity, have less phenylalanine, tyrosine or calcium than the natural aliment of *L. schreiberi*, which might have lead to lower melanin production. But corticosterone can decrease melanin-based coloration by specific glucocorticoid receptors in melanophores, leading to melanosome aggregation within melanophores and/or reduction of melanin concentration (Greenberg, 2002; Ducrest et al., 2008; Roulin et al., 2008). Like melanophores, iridophores have similar cell receptors that belong to the same receptor superfamily as glucocorticoids receptors (Evans, 1988; Miwa &

Yamano, 1999), which may influence spacing of iridophores or spacing of reflective platelets inside. Females of *Sceloporus virgatus* react to corticosterone with smaller and less colorful throat patches (Weiss et al. 2013). But , surprisingly, in our study, this captivity stress did not affect mother condition, nor immune response after egg laying, which was, however, expected given that corticosterone is an energy mobilizing hormone (Romero 2002; Moore and Jessop 2003) and that long-termed increased corticosterone levels by chronic stress have suppressive effects on the immune system (Dhabhar and Mcewen 1997; Dhabhar 2000). However, corticosterone did not affect post-parturition body mass of corticosterone implanted female *L. vivipara*, but in this experiment offspring body size was neither affected. Furthermore, the immune system was suppressed by corticosterone only when animals were energetically compromised in female tree lizard (*Urosaurus ornatus*) (French et al. 2007).

In summary, we found that structural, carotenoid and melanin-based coloration of females together may indicate both their own quality and that of their offspring. However, in our study, color measures before mating was not possible, only after egg laying. We do not know whether and how antioxidant investment in coloration may change under the need of investing on vitellogenesis. However, after egg laying females still had considerable coloration, which may suggest that females do not use the same investment in coloration and later into the eggs. A more intense coloration rather would show a better nutrient supply for both the body of females and their offspring. Other important requirement for future studies is that, although we suggest the base of possible mutual mate choice, we need to prove that males indeed prefer these color signals of females and that this choice increase their fitness.

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Ethical standards

The experiments enforced all the present Spanish laws and were performed under license (permit number: 10/142790.9/11) from the Environmental Organisms of Madrid Community where they were carried out.

Reference

Amundsen T (2000) Why are female birds ornamented? Trends Ecol Evol 15:149–155

Belliure J, Smith L, Sorci G (2004) Effect of testosterone on T cell-mediated immunity in two species of Mediterranean lacertid lizards. J Exp Zool A 301:411–418

Berglund A, Rosenqvist G (2001) Male pipefish prefer ornamented females. Anim Behav 61:345–350

Biard C, Surai PF, Møller AP (2005) Effects of carotenoid availability during laying on reproduction in the blue tit. Oecologia 144:32–44

Bleu J, Massot M, Haussy C, Meylan S (2012) Experimental litter size reduction reveals costs of gestation and delayed effects on offspring in a viviparous lizard. Proc R Soc Lond B 279:489–498

Blount JD, Houston DC, Møller AP (2000) Why egg yolk is yellow. Trends Ecol Evol 15:47–49

Blount JD, Surai PF, Nager RG, Houston DC, Møller AP, Trewby ML, Kennedy MW (2002) Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. Proc R Soc Lond B 269:29–36

Bonduriansky R (2001) The evolution of male mate choice in insects: a synthesis of ideas and evidence. Biol Rev 76:305–339

Boulinier T, Staszewski V (2008) Maternal transfer of antibodies: raising immuno-ecology issues. Trends Ecol Evol 23:282–288

Burley N (1977) Parental investment, mate choice, and mate quality. P Natl Acad Sci USA 74:3476–3479

Caley MJ, Schwarzkopf L, Shine R (2001) Does total reproductive output evolve independently of offspring size? Evolution 55:1245-1248

Chew BP, Park JS (2004) Carotenoid action on the immune response. J Nutr 134:257–261

Civantos E, Forsman A (2000) Determinants of survival in juvenile *Psammodromus algirus* lizards. Oecologia 124:64–72

Civantos E, Salvador A, Veiga JP (1999) Body size and microhabitat affect winter survival of hatchling *Psammodromus algirus* lizards. Copeia 1999:1112–1117

Cloudsley-Thompson JL (1999) Multiple factors in the evolution of animal coloration. Naturwissenschaften 86:123–132

Clutton-Brock T (2009) Sexual selection in females. Anim Behav 77:3–11

Commins MA, Goodger BV, Waltisbuhl DJ, Wright IG (1988*) Babesia bovis*: studies of parameters influencing microvascular stasis of infected erythrocytes. Res Vet Sci 44:226–228

Cornwallis CK, Birkhead TR (2007) Experimental evidence that female ornamentation increases the acquisition of sperm and signals fecundity. Proc R Soc Lond 274:583–590

Costanzo JP, Grenot C, Lee RE (1995) Supercooling, ice inoculation and freeze tolerance in the European common lizard, *Lacerta vivipara*. J Comp Physiol B 165:238–244

Cuthill IC, Bennett ATD, Partridge JC, Maier EJ (1999) Plumage reflectance and the objective assessment of avian sexual dichromatism. Am Nat 153:183–200

Das BS, Thurnham DI, Das DB (1996) Plasma alpha-tocopherol, retinol, and carotenoids in children with falciparum malaria. Am J Clin Nutr 64:94-100

Deger S, Deger Y, Bicek K, Ozdal N, Gul A (2009) Status of lipid peroxidation, antioxidants, and oxidation products of nitric oxide in equine babesiosis: status of antioxidant and oxidant in equine babesiosis. J Equine Vet Sci 29:743-747

Dewsbury DA (1982) Ejaculate cost and male choice. Am Nat 119:601–610

Dhabhar FS (2000) Acute stress enhances while chronic stress suppresses skin immunity: the role of stress hormones and leukocyte trafficking. Ann NY Acad Sci 917:876–893

Dhabhar FS, Mcewen BS (1997) Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: A potential role for leukocyte trafficking. Brain Behav Immun 11:286–306

Dreiss AN, Roulin A (2010) Age-related change in melanin-based coloration of Barn owls (*Tyto alba*): females that become more female-like and males that become more male-like perform better. Biol J Linn Soc 101:689–704

Ducrest AL, Keller L, Roulin A (2008) Pleiotropy in the melanocortin system, coloration and behavioural syndromes. Trends Ecol Evol 23:502–510

Endler JA (1990) On the measurement and classification of colour in studies of animal colour patterns. Biol J Linn Soc 41:315–352

Eriksen MS, Haug A, Torjesen PA, Bakken M (2003) Prenatal exposure to corticosterone impairs embryonic development and increases fluctuating asymmetry in chickens (*Gallus gallus domesticus*). Br Poult Sci 44:690–697

Evans RM (1988) The steroid and thyroid hormone receptor superfamily. Science 240:889–895

Fitzpatrick S, Berglund A, Rosenqvist G (1995) Ornaments or offspring: costs to reproductive success restrict sexual selection processes. Biol J Linn Soc 55:251–260

Fleishman LJ, Loew ER, Leal M (1993) Ultraviolet vision in lizards. Nature 365:397

Freeman-Gallant CR, Schneider RL, Taff CC, Dunn PO, Whittingham LA (2014) Contrasting patterns of selection on the size and coloration of a female plumage ornament in common yellowthroats. J Evol Biol 27:982–991

French SS, McLemore R, Vernon B, Johnston GI, Moore MC (2007) Corticosterone modulation of reproductive and immune systems trade-offs in female tree lizards: long-term corticosterone manipulations via injectable gelling material. J Exp Biol 210:2859–2865

Gasparini J, McCoy KD, Haussy C, Tveraa T, Boulinier T (2001) Induced maternal response to the Lyme disease spirochaete *Borrelia burgdorferi sensu lato* in a colonial seabird, the kittiwake *Rissa tridactyla*. Proc R Soc Lond B 268:647–650

George SB, Lawrence JM, Lawrence AL, Smiley J, Plank L (2001) Carotenoids in the adult diet enhance egg and juvenile production in the sea urchin *Lytechinus variegatus*. Aquaculture 199:353–369

Greenberg N (2002) Ethological aspects of stress in a model lizard, *Anolis carolinensis*. Integr Comp Biol 42:526–540

Grenot CJ, Garcin L, Dao J, Hérold J-P, Fahys B, Tséré-Pagès H (2000) How does the European common lizard, *Lacerta vivipara*, survive the cold of winter? Comp Biochem Phys A 127:71–80

Griggio M, Zanollo V, Hoi H (2010) Female ornamentation, parental quality, and competitive ability in the rock sparrow. J Ethol 28:455–462

Grill CP, Rush VN (2000) Analysing spectral data: comparison and application of two techniques. Biol J Linn Soc 69:121–138

Hayward LS, Wingfield JC (2004) Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. Gen Comp Endocrinol 135:365–371

Hegyi G, Garamszegi LZ, Eens M, Török J (2008) Female ornamentation and territorial conflicts in collared flycatchers (Ficedula albicollis). Naturwissenschaften 95:993–996

Heiblum R, Arnon E, Chazan G, Robinzon B, Gvaryahu G, Snapir N (2001) Glucocorticoid administration during incubation: embryo mortality and posthatch growth in chickens. Poult Sci 80:1357–1363

Heinsohn R, Legge S, Endler JA (2005) Extreme reversed sexual dichromatism in a bird without sex role reversal. Science 309:617–619

Henderson LJ, Heidinger BJ, Evans NP, Arnold KE (2013) Ultraviolet crown coloration in female blue tits predicts reproductive success and baseline corticosterone. Behav Ecol 24:1299–1305

Ibáñez A, Marzal A, López P, Martín J (2013) Sexually dichromatic coloration reflects size and immunocompetence in female Spanish terrapins, *Mauremys leprosa*. Naturwissenschaften 100:1137–1147

Itzkowitz M, Draud MJ, Barnes JL, Haley M (1998) Does it matter that male beaugregory damselfish have a mate preference? Behav Ecol Sociobiol 42:149–155

Izquierdo MS, Fernandez-Palacios H, Tacon AGJ (2001) Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197:25–42

Jawor JM, Gray N, Beall SM, Breitwisch R (2004) Multiple ornaments correlate with aspects of condition and behaviour in female northern cardinals, *Cardinalis cardinalis*. Anim Behav 67:875–882

Johnsen A, Delhey K, Andersson S, Kempenaers B (2003) Plumage colour in nestling blue tits: sexual dichromatism, condition dependence and genetic effects. Proc R Soc Lond B 270:1263–1270

Johnsen TS, Hengeveld JD, Blank JL, Yasukawa K, Nolan V (1996) Epaulet brightness and condition in female red-winged blackbirds. The Auk:356–362

Johnston TA, Wiegand MD, Leggett WC, Pronyk RJ, Dyal SD, Watchorn KE, Kollar S, Casselman JM (2007) Hatching success of walleye embryos in relation to maternal and ova characteristics. Ecol Freshw Fish 16:295–306

Johnstone RA, Reynolds JD, Deutsch JC (1996) Mutual mate choice and sex differences in choosiness. Evolution 50:1382–1391

Kelly RJ, Murphy TG, Tarvin KA, Burness G (2012) Carotenoid-based ornaments of female and male American goldfinches (*Spinus tristis*) show sex-specific correlations with immune function and metabolic rate. Physiol Biochem Zool 85:348–363

Kemp DJ, Rutowski RL (2007) Condition dependence, quantitative genetics, and the potential signal content of iridescent ultraviolet butterfly coloration. Evolution 61:168–183

Kokko H, Monaghan P (2001) Predicting the direction of sexual selection. Ecol Lett 4:159–165

Kopena R, López P, Martín J (2014a) Relative contribution of dietary carotenoids and vitamin E to visual and chemical sexual signals of male Iberian green lizards: an experimental test. Behav Ecol Sociobiol 68:571–581

Kopena R, López P, Martín J (2014b) What are carotenoids signaling? Immunostimulatory effects of dietary vitamin E, but not of carotenoids, in Iberian green lizards. Naturwissenschaften 101:1107–1114

Kuriyama T, Miyaji K, Sugimoto M, Hasegawa M (2006) Ultrastructure of the dermal chromatophores in a lizard (Scincidae: *Plestiodon latiscutatus*) with conspicuous body and tail coloration. Zool Sci 23:793-799

Lande R (1980) Sexual dimorphism, sexual selection, and adaptation in polygenic characters. Evolution 34:292–305

Ligon RA, McCartney KL (2016) Biochemical regulation of pigment motility in vertebrate chromatophores: A review of physiological color change mechanisms. Curr Zool 62:237–252

Loew ER, Fleishman LJ, Foster RG, Provencio I (2002) Visual pigments and oil droplets in diurnal lizards a comparative study of Caribbean anoles. J Exp Biol 205:927–938

Marco A (2015) Lagarto verdinegro - *Lacerta schreiberi*. In: Salvador A, Marco A (eds) Enciclopedia Virtual de los Vertebrados Españoles. Museo Nacional de Ciencias Naturales, Madrid, http://www.vertebradosibericos.org

Marco A, Pérez-Mellado V (1999) Mate guarding, intrasexual competition and mating success in males of the non-territorial lizard *Lacerta schreiberi*. Ethol Ecol Evol 11:279–286

Martín J, López P (2009) Multiple color signals may reveal multiple messages in male Schreiber's green lizards, *Lacerta schreiberi*. Behav Ecol Sociobiol 63:1743–1755

Martin LB, Han P, Lewittes J, Kuhlman JR, Klasing KC, Wikelski M (2006) Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct Ecol 20:290–299

Mashaly MM (1991) Effect of exogenous corticosterone on chicken embryonic development. Poult Sci 70:371–374

Mäthger LM, Hanlon RT (2007) Malleable skin coloration in cephalopods: selective reflectance, transmission and absorbance of light by chromatophores and iridophores. Cell Tissue Res 329:179–186

McGraw KJ (2008) An update on the honesty of melanin-based color signals in birds. Pigm Cell Melanoma R 21:133–138

McGraw KJ, Adkins-Regan E, Parker RS (2005) Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. Naturwissenschaften 92:375–380

Miwa S, Yamano K (1999) Retinoic acid stimulates development of adult-type chromatophores in the flounder. J Exp Zool 284:317–324

Montgomerie R, Hill GE, McGraw KJ (2006) Analyzing colors. In: Hill GE, McGraw KJ (eds) Bird coloration vol 1. Mechanisms and measurements. Harvard University Press, Cambridge, pp 90–147

Moore IT, Jessop TS (2003) Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. Horm Behav 43:39–47

Morales J, Moreno J, Merino S, Sanz JJ, Tomás G, Arriero E, Lobato E, Martínez-de la Puente J (2007) Female ornaments in the Pied Flycatcher *Ficedula hypoleuca*: associations with age, health and reproductive success. Ibis 149:245-254

Nordeide JT (2002) Do male sticklebacks prefer females with red ornamentation? Can J Zool 80:1344–1349

Nordeide JT, Rudolfsen G, Egeland ES (2006) Ornaments or offspring? Female sticklebacks (*Gasterosteus aculeatus* L.) trade off carotenoids between spines and eggs. J Evol Biol 19:431–439

Owens IP, Thompson DB (1994) Sex differences, sex ratios and sex roles. Proc R Soc Lond B 258:93–99

Parker GA (1982) Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. J Theor Biol 96:281–294

Parker GA (1983) Mate quality and mating decisions. In: Bateson P (ed) Mate Choice. Cambridge University Press, Cambridge, pp 141:166

Parker GA, Simmons LW (1996) Parental investment and the control of sexual selection: predicting the direction of sexual competition. Proc R Soc Lond B 263:315–321

Parker GA, Ball MA, Stockley P, Gage MJG (1996) Sperm competition games: individual assessment of sperm competition intensity by group spawners. Proc R Soc Lond B 263:1291–1297

Parker GA, Ball MA, Stockley P, Gage MJG (1997) Sperm competition games: a prospective analysis of risk assessment. Proc R Soc Lond B 264:1793–1802

Peters A, Denk AG, Delhey K, Kempenaers B (2004) Carotenoid-based bill colour as an indicator of immunocompetence and sperm performance in male mallards. J Evol Biol 17:1111–1120

Potti J, Merino S (1996) Decreased levels of blood trypanosome infection correlate with female expression of a male secondary sexual trait: implications for sexual selection. Proc R Soc Lond B 263:1199–1204

Potti J, Canal D, Serrano D (2013) Lifetime fitness and age-related female ornament signalling: evidence for survival and fecundity selection in the pied flycatcher. J Evol Biol 26:1445–1457

Reguera S, Zamora-Camacho FJ, Moreno-Rueda G (2014) The lizard *Psammodromus algirus* (Squamata: Lacertidae) is darker at high altitudes. Biol J Linn Soc 112:132–141

Remeš V, Matysioková B (2013) More ornamented females produce higher-quality offspring in a socially monogamous bird: an experimental study in the great tit (*Parus major*). Front Zool 10:14

Roca V, Galdón MA (2010) Haemogregarine blood parasites in the lizards *Podarcis bocagei* (Seoane) and P. carbonelli (Pérez-Mellado)(Sauria: Lacertidae) from NW Portugal. Syst Parasitol 75:75-79

Roff DA (1992) The Evolution of Life Histories. Chapman & Hall, New York

Romero LM (2002) Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. Gen Comp Endocrinol 128:1–24

Roulin A, Almasi B, Rossi-Pedruzzi A, Ducrest A-L, Wakamatsu K, Miksik I, Blount JD, Jenni-Eiermann S, Jenni L (2008) Corticosterone mediates the condition-dependent component of melanin-based coloration. Anim Behav 75:1351–1358

Roulin A, Altwegg R, Jensen H, Steinsland I, Schaub M (2010) Sex-dependent selection on an autosomal melanic female ornament promotes the evolution of sex ratio bias. Ecol Lett 13:616–626

Rubolini D, Romano M, Boncoraglio G, Ferrari RP, Martinelli R, Galeotti P, Fasola M, Saino N (2005) Effects of elevated egg corticosterone levels on behavior, growth, and immunity of yellow-legged gull (*Larus michahellis*) chicks. Horm Behav 47:592–605

Saenko SV, Teyssier J, Van Der Marel D, Milinkovitch MC (2013) Precise colocalization of interacting structural and pigmentary elements generates extensive color pattern variation in *Phelsuma* lizards. BMC biol 11:105.

Salaberria C, Muriel J, de Luna M, Gil D, Puerta M (2013) The PHA test as an indicator of phagocytic activity in a passerine bird. PloS One 8:e84108

Saleh MA (2009) Erythrocytic oxidative damage in crossbred cattle naturally infected with *Babesia bigemina*. Res Vet Sci 86:43–48

Savalli UM, Fox CW (1999) The effect of male mating history on paternal investment, fecundity and female remating in the seed beetle *Callosobruchus maculatus*. Funct Ecol 13:169–177

Scheuerlein A, Van't Hof T, Gwinner E (2001) Predators as stressors? Physiological and reproductive consequences of predation risk in tropical stonechats (*Saxicola torquata axillaris*). Proc R Soc Lond B 268:1575–1582

Schwabl H (1996) Maternal testosterone in the avian egg enhances postnatal growth. Comp Biochem Physiol A 114:271–276

Schwarzkopf L (1992) Annual variation of litter size and offspring size in a viviparous skink. Herpetologica 48:390-395

Schwarzkopf L, Blows MW, Caley MJ (1999) Life-history consequences of divergent selection on egg size in *Drosophila melanogaster*. Am Nat 29:333-340

Siefferman L, Hill GE (2005) Evidence for sexual selection on structural plumage coloration in female eastern bluebirds (*Sialia sialis*). Evolution 59:1819–1828

Silverin B (1998) Behavioural and hormonal responses of the pied flycatcher to environmental stressors. Anim Behav 55:1411–1420

Sinervo B (1990) The evolution of maternal investment in lizards: an experimental and comparative analysis of egg size and its effects on offspring performance. Evolution 44:279-294.

Skarstein F, Folstad I (1996) Sexual dichromatism and the immunocompetence handicap: an observational approach using Arctic charr. Oikos 76:359–367

Smits JE, Bortolotti GR, Tella JL (1999) Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. Funct Ecol 13:567–572

Speake BK, Surai PF, Gore M (2001) Lipid composition, fatty acid profiles, and lipid-soluble antioxidants of eggs of the Hermann's tortoise (*Testudo hermanni boettgeri*). Zoo Biol 20:75–87

Squires EL, Pickett BW, Amann RP (1978) Effect of successive ejaculation on stallion seminal characteristics. J Rep Fer S 27:7–12

Stearns SC (1992) The Evolution of Life Histories. Oxford University Press, Oxford

Steiger S (2013) Bigger mothers are better mothers: disentangling size-related prenatal and postnatal maternal effects. Proc R Soc B 280:20131225

Surai PF, Speake BK, Sparks NHC (2001a) Carotenoids in avian nutrition and embryonic development. 2. Antioxident properties and discrimination in embryonic tissues. J Poult Sci 38:117–145

Surai PF, Speake BK, Sparks NHC (2001b) Carotenoids in avian nutrition and embryonic development. 1. Absorption, availability and levels in plasma and egg yolk. J Poult Sci 38:1–27

Szigeti B, Török J, Hegyi G, Rosivall B, Hargitai R, Szöllősi E, Michl G (2007) Egg quality and parental ornamentation in the blue tit Parus caeruleus. J Avian Biol 38:105–112

Telford SR (2008) Hemoparasites of the reptilia. Color atlas and text. CRC Press, Boca Raton

Thompson MB, Speake BK (2002) Energy and nutrient utilisation by embryonic reptiles. Comp Biochem Phys A 133:529–538

Tobler M, Healey M, Wilson M, Olsson M (2011) Basal superoxide as a sex-specific immune constraint. Biol Lett 7:906–908

Tomás G, Merino S, Martínez J, Moreno J, Sanz JJ (2005). Stress protein levels and blood parasite infection in blue tits (Parus caeruleus): a medication field experiment. Ann Zool Fenn 42:45–56

Vinkler M, Schnitzer J, Munclinger P, Albrecht T (2012) Phytohaemagglutinin skin-swelling test in scarlet rosefinch males: low-quality birds respond more strongly. Anim Behav 83:17–23

Warner DA, Shine R (2009) Maternal and environmental effects on offspring phenotypes in an oviparous lizard: do field data corroborate laboratory data? Oecologia 161:209–220

Weiss SL (2006) Female-specific color is a signal of quality in the striped plateau lizard (*Sceloporus virgatus*). Behav Ecol 17:726–732

Weiss SL, Kennedy EA, Bernhard JA (2009) Female-specific ornamentation predicts offspring quality in the striped plateau lizard, *Sceloporus virgatus*. Behav Ecol 20:1063-1071

Weiss SL, Kennedy EA, Safran RJ, McGraw KJ (2011) Pterin-based ornamental coloration predicts yolk antioxidant levels in female striped plateau lizards (*Sceloporus virgatus*). J Anim Ecol 80:519–527

Weiss SL, Mulligan EE, Wilson DS, Kabelik D (2013) Effect of stress on female-specific ornamentation. J Exp Biol 216:2641–2647

Wiegand MD (1996) Composition, accumulation and utilization of yolk lipids in teleost fish. Rev Fish Biol Fish 6:259–286

Wright DS, Pierotti MER, Rundle HD, McKinnon JS (2015) Conspicuous Female Ornamentation and Tests of Male Mate Preference in Threespine Sticklebacks (*Gasterosteus aculeatus*). PLoS ONE 10(3):e0120723

Resumen extenso en español

Función y evolución de señales múltiples en procesos de selección sexual del lagarto verdinegro (*Lacerta schreiberi*)

Introducción

La elección de pareja es un proceso básico para los animales que se reproducen sexualmente. La elección de pareja puede basarse en beneficios directos, en los que los machos (o raramente las hembras) ofrecen recursos al otro sexo (por ej., cuidado parental, "regalos", etc.), o beneficios indirectos, donde sólo se ofrece a las hembras una dotación genética que aumentará el "fitness" futuro de la hembra gracias a los buenos genes y/o atractivo de su descendencia. Los machos tienen que señalar sus beneficios directos o indirectos a las hembras para ser elegidos para la reproducción, aumentando así su propio "fitness". Estas señales son la base de la comunicación sexual entre machos y hembras, que puede ser estable a largo plazo si los beneficios del señalador y del receptor exceden los costos de comunicación. La señalización es costosa por el tiempo y la energía que consume, y además, puede aumentar el riesgo de depredación mediante el aumento de la conspicuidad. Sin embargo, la señalización vale la pena si el animal puede aumentar su fitness al tener éxito en la adquisición de pareja. Desde el punto de vista del receptor, la evaluación de la señal necesita tiempo y puede aumentar los costes cerebrales o requerir el desarrollo de sistemas sensoriales adecuados, pero los beneficios directos y/o indirectos también pueden aumentar su propio fitness.

Las exhibiciones sexuales que los animales usan en la elección de pareja pueden ser muy complejas, tales como el uso de ornamentos con coloraciones brillantes, cantos elaborados y/o exhibiciones conspicuas de cortejo. Hay varias teorías para explicar la

evolución y la función de las señales complejas. Las señales complejas pueden consistir en varios componentes que sólo pueden interpretarse cuando se perciben juntos (señales multicomponentes), o ser señales en las que cada componente de la señal puede provocar por sí mismo una respuesta comportamental (señales múltiples), o en algunos casos especiales los diferentes tipos de señales pueden ser detectadas por diferentes sistemas sensoriales (señales multimodales). Diferentes señales pueden reflejar diferentes aspectos de la calidad de una pareja potencial o bien todas las señales pueden indicar la misma calidad, lo que permite al receptor estimar con mayor precisión la calidad del individuo que emite la señal y hace más difícil que la señal no sea honesta. Las señales multicomponentes pueden provocar reacciones más fuertes en el receptor que una única señal debido a una mayor detectabilidad, reconocimiento, discriminación y memorización. Los distintos componentes de las señales múltiples pueden estar relacionados y pueden amplificarse entre sí mediante interacciones.

La mayoría de las veces las señales sexuales de los lagartos son visuales y olfatorias. Las señales de coloración basadas en carotenoides, melanina y estructurales tienen costes de producción o tienen componentes que deben ser adquiridos de la dieta y también tienen costes de transformación. Además, la presencia de costes para el sistema inmune y el anti-oxidativo pueden permitir la fiabilidad y el mantenimiento de la señal. En la comunicación química, las señales químicas con múltiples compuestos pueden tener diferentes múltiples mensajes. Las señales químicas de los lagartos consisten en compuestos lipofílicos y proteínas. Las proteínas probablemente tienen un papel en el reconocimiento interespecífico, pero los compuestos lipofílicos, con una mayor variabilidad interindividual, pueden ser más importantes para la comunicación en un contexto reproductivo. La fuente de los componentes lipídicos es el metabolismo general y, por lo tanto, las características de las señales químicas pueden estar directamente relacionadas con la salud y la condición del animal dando como resultado una señal honesta. Por ejemplo, el α-tocoferol (=vitamina E) tiene funciones importantes tanto en el sistema inmune como en el antioxidante. La composición de las señales químicas puede estar relacionada con la edad/tamaño, características morfológicas, carga de parásitos, estado de salud, condición, atractivo o

estatus de dominancia en diferentes especies de lagartijas, lo que confirma el papel relevante de las señales químicas en las interacciones intra e intersexuales de las lagartijas. La evolución de las señales químicas probablemente se explica por una búsqueda sensorial sesgada de los productos químicos que también se encuentran en los alimentos, pero la fiabilidad de la señal puede permitir posteriormente su mantenimiento. Los lagartos pueden usar señales químicas y visuales como señales multimodales, explotando diferentes canales sensoriales, pero diferentes componentes de las señales de color o químicas también pueden tener mensajes diferentes.

Las Investigaciones sobre señales sexuales suelen basarse en los machos, pero las hembras también pueden tener señales sexuales. Según una teoría, los ornamentos de las hembras serían sólo el resultado de un proceso no adaptativo de correlación genética intersexual debido al efecto de la selección sexual o natural sobre el genoma de los machos, que también es heredado por las hembras. En este caso, los ornamentos conspicuos tienen un efecto negativo sobre la posibilidad de supervivencia y la fecundidad de las hembras. Pero hoy en día se ha demostrado que los adornos de las hembras pueden indicar calidad, informando acerca de su estado de salud, condición y posible fecundidad e inversión en su descendencia. Aunque la correlación genética intersexual y la selección directa dan predicciones opuestas sobre la relación entre señales y fitness de las hembras, estos no son fenómenos mutuamente excluyentes. Los rasgos vestigiales resultado de la correlación genética pueden ser una base para desarrollar otros ornamentos por selección sexual. Además, la cooperación de estos dos procesos puede ocurrir; Las parejas femeninas atractivas pueden resultar beneficiosas para los machos al producir no sólo hijas vistosas, sino también hijos más atractivos y viceversa.

Además, el mantenimiento de señales conspicuas tiene otro coste, el aumento del riesgo de depredación. Los animales pueden adaptarse al riesgo de depredación al disminuir la intensidad de la señal, o bien, para compensar este aumento de riesgo, los animales pueden modificar su comportamiento. La compensación del comportamiento en las especies sexualmente dicromáticas puede manifestarse en que el sexo más ornamentado permita que

el depredador se acerque menos que lo que permite el otro género con mejores propiedades crípticas. La búsqueda de pareja y el aumento de testosterona también pueden cambiar el comportamiento de compensación. Por lo tanto, la adaptación al riesgo real de depredación y los cambios de comportamiento antidepredador pueden ser capaces de compensar (al menos hasta cierto nivel) el coste de las señales conspicuas.

Objetivos

El objetivo principal de esta tesis es estudiar la función, evolución y mantenimiento de las señales sexuales que múltiples utilizan los lagartos verdinegros (*Lacerta schreiberi*) en los procesos de selección sexual y los efectos de la presión de la selección natural sobre estas señales en las estrategias antidepredatorias de los lagartos. Los siguientes seis capítulos lo elaborarán a través de los siguientes objetivos concretos.

Objetivo I. Las señales sexuales conspicuas, la búsqueda de pareja y la guarda de parejas pueden aumentar en gran medida el riesgo de depredación en los machos de especies sexualmente dicromaticas. En este estudio se examina si las diferencias intersexuales en la coloración y las exigencias reproductivas afectan al comportamiento preventivo de riesgo (es decir, a la distancia total al refugio potencial más cercano que el lagarto mantiene antes de un ataque) y a las decisiones de escape activo en una situación de emergencia cuando se produce un ataque (es decir, a la distancia entre el lagarto y el depredador cuando el lagarto empieza a huir) o si solo las condiciones ambientales actuales influyen en el comportamiento antidepredatorio. (**Capítulo I**)

Objetivo II. Los costes de las señales sexuales basadas en carotenoides están bien investigados en aves, pero son poco conocidos en los vertebrados inferiores. Los costes de desarrollo y mantenimiento de otras señales visuales y químicas han sido aun menos investigados en todo el reino animal. En los tres estudios siguientes investigamos la relación

entre el sistema antioxidante y el sistema inmunológico con las señales sexuales múltiples altamente elaboradas, que incluyen señales visuales de coloración, basadas en carotenoides, melanina y en coloración estructural, y señales químicas.

A, Este estudio examina si la suplementación en la dieta de antioxidantes, como los carotenoides pigmentarios y la vitamina E no pigmentaria, influye en diferentes señales sexuales visuales y químicas. (**Capítulo II**)

B, Este estudio examina los efectos de los carotenoides y la vitamina E en la dieta sobre la condición corporal y la respuesta inmune inducida por PHA. (**Capítulo III)**

C, En este estudio se investiga si los costes de una activación inmune mediante LPS afectan a la expresión de señales sexuales de diferentes tipos (visual y químico), y los efectos interactivos con una suplementación en la dieta de un importante antioxidante, la vitamina E. (**Capítulo IV**)

Objetivo III. Los patrones de movimiento de los animales pueden revelar las relaciones sociales y el comportamiento de apareamiento de la especie. En este estudio se investigan los tamaños y el solapamiento de las áreas de campeo (dominios vitales) de los lagartos machos y hembras y su relación con las características de las señales sexuales para analizar cómo el estatus de dominancia de los machos y el atractivo de ambos sexos pueden influir en su uso espacial. **(Capítulo V)**

Objetivo IV. Las señales sexuales de las hembras han sido menos investigadas en comparación con las de los machos. Aquí se comparan las características de color de ambos sexos y, además, se examina si la coloración basada en carotenoides, melanina o en coloración estructural de las hembras de *L. schreiberi* pueden tener una función de señalización de algún parámetro de la salud o de componentes del potencial reproductivo de estas hembras. (**Capítulo VI**)

Resultados

Comportamiento antidepredatorio de L. schreiberi

Capítulo I. Examinamos el papel relativo del microhábitat, la posición del depredador y el refugio, la estación y el sexo al determinar (i) la distancia al refugio más cercano que los lagartos mantienen antes de un ataque (distancia al refugio) y (ii) la distancia que los lagartos permiten que se acerque un depredador simulado antes de huir (distancia de iniciación de huida, FID). La distancia al refugio no fue afectada por las variables estudiadas. Sin embargo, la FID se relacionó positivamente con la distancia al refugio en microhábitats con hierba, pero no sobre sustratos rocosos. Además, la distancia al refugio y el ángulo de escape interactuaron independientemente del sustrato: los lagartos permitieron a los depredadores acercarse más cuando los refugios estaban cerca o cuando los lagartos tuvieron que huir hacia el depredador. En contraste, ni la época de apareamiento ni el sexo afectaron a la FID. Sugerimos que la estrategia de escape de *L. schreiberi* está determinada más por el ambiente físico que por el sexo o la condición reproductiva.

Efectos de un desafío sobre el sistema antioxidante e inmune sobre las señales sexuales, sistema inmune y condición corporal

Capítulo II. Los resultados indicaron que tanto la suplementación en la dieta de carotenoides como de vitamina E aumentaron la expresión de algunas señales visuales (coloración de garganta y pecho, pero no la dorsal) en comparación con los controles. Sin embargo, diferentes rasgos fueron afectados diferencialmente y, en muchos casos, la adición de vitamina E, sola o en combinación con carotenoides, tuvo un mayor efecto sobre la expresión de coloración que la adición de carotenoides solos, incluso para ornamentos dependientes de carotenoides. Nuestros resultados apoyan la idea de que se necesitan otros antioxidantes no pigmentarios, como la vitamina E, además de los carotenoides para aumentar la expresión de la coloración de los lagartos verdinegros, *L. schreiberi*. Por lo tanto, la coloración podría reflejar indirectamente los niveles de antioxidantes no pigmentarios. En cambio, un aumento

de la vitamina E antioxidante no pigmentaria en la dieta se reflejó directamente en las señales químicas. Debido a la concordancia observada entre las señales visuales y químicas, sugerimos que ambos tipos de señales pueden ser utilizadas en diferentes contextos, transmitiendo mensajes similares pero en diferentes canales sensoriales.

Capítulo III. Los lagartos que fueron suplementados con vitamina E, sola o combinada con carotenoides, tuvieron mayores respuestas inmunes que los lagartos control, pero los animales suplementados sólo con carotenoides tuvieron respuestas inmunes más bajas que los lagartos suplementados con vitamina E y no difirieron de los controles. Estos resultados apoyan la hipótesis de que los carotenoides en el lagarto verdinegro no son eficaces como inmunoestimulantes, pero que pueden estar señalando visualmente los efectos inmunoestimuladores de la vitamina E no pigmentaria. En contraste, los lagartos suplementados sólo con carotenoides tienen mayores incrementos de la condición corporal, lo que sugiere que los carotenoides pueden seguir siendo importantes para mejorar la condición.

Capítulo IV. Los lagartos que sufrieron un desafío inmune no disminuyeron la intensidad de las señales sexuales basadas en carotenoides o el contenido de vitamina E de las señales químicas, sino que además respondieron con una mayor intensidad de las señales UV-azules basadas en melanina (es decir, la coloración de la garganta). Los machos que sufren un desafío inmune podrían estar tratando de maximizar sus posibilidades de reproducción presentes aumentando la intensidad de la señal de la garganta en una situación donde las expectativas a largo plazo de supervivencia y reproducción futura son bajas. Sorprendentemente, la vitamina E, sola o combinada con un desafío inmune, disminuyó la coloración basada en carotenoides pero aumentó la coloración basada en melanina, lo cual es opuesto a nuestros resultados anteriores cuando las condiciones climáticas en el año de estudio eran muy diferentes. Es posible que esto se explique si las hembras pueden cambiar su preferencia por las señales de los machos entre años según qué tipo de señal puede indicar con mayor fiabilidad la calidad de los machos en diferentes condiciones ambientales.

Uso del espacio de L. schreiberi

Capítulo V. Se encontró que los machos con más rasgos de dominancia (cabeza relativamente más grande o coloración azul-UV de la garganta más intensa) usan áreas de campeo más grandes que se superponen con las de más machos y más hembras, y con más machos dominantes. Mientras que los machos menos dominantes usan áreas más pequeñas que evitan a las de estos machos dominantes. Los resultados de los solapamientos entre machos y hembras claramente dibujan dos estrategias reproductivas diferentes en cuanto al uso del espacio: Los machos dominantes usan áreas más grandes, probablemente tratando de aumentar el éxito reproductivo al solapar y aparearse con más hembras. En contraste, los machos menos dominantes usan áreas más pequeñas y prefieren guardar parejas, accediendo a menos hembras pero de mayor calidad (con coloración dorsal con más UV y más verde). Las hembras que utilizan áreas más grandes solapan con más machos, pero las hembras que solapan con machos con más vitamina E en sus señales químicas (de alta calidad) usan áreas más pequeñas. Las diferentes estrategias de apareamiento de los machos pueden ser estrategias evolutivamente estables que proporcionan beneficios para ambos tipos de machos. Un éxito reproductivo similar podría mantener la honestidad de las señales y el sistema de señales sexuales múltiples.

Señales sexuales de las hembras de L. schreiberi

Capítulo VI. Al investigar el dicromatismo sexual, encontramos que los machos tenían una coloración UV y azul más saturada en la garganta y las espaldas más verdes. Las hembras eran más brillantes en todas las partes del cuerpo y, sorprendentemente, tenían un pecho más amarillento con mayor croma de carotenoides que los machos. Investigando el papel potencial de las señales sexuales de la calidad de las hembras, encontramos que las hembras con menos parásitos en la sangre tenían una coloración UV y azul más intensa de la garganta, que podría ser causada por el efecto positivo de los antioxidantes en la disposición de plaquetas reflectantes en los iridóforos. Además, las hembras con menos garrapatas y respuestas inmunes más fuertes tenían coloración dorsal UV y verde más intensa (probablemente producida por más carotenoides). Investigando los costes reproductivos de

las hembras, encontramos que la vitelogénesis tenía efectos graves en la condición, pero no en la respuesta inmune. La condición de la madre después de la puesta de los huevos disminuyó al aumentar el tamaño relativo de la puesta. Después de la puesta, la respuesta inmune basada en PHA dependió positivamente sólo del tamaño de la madre. El número de huevos puestos fue determinado solamente por el tamaño de la madre, mientras que su carga de parásitos no afectó al tamaño absoluto o relativo de la puesta.

La coloración de la madre no predijo el tamaño relativo de la puesta, pero las hembras con más coloración dorsal saturada tuvieron mayor éxito en la eclosión y más crías. Además, las hembras con coloración dorsal más saturada pusieron sus huevos antes. Los huevos de las hembras con pechos más amarillentos tuvieron un tiempo de incubación más largo, sin embargo, las hembras con coloración dorsal más verde tuvieron huevos con tiempos de incubación un poco más cortos. Los carotenoides y otras inversiones de antioxidantes en la yema pueden aumentar la supervivencia de los huevos y la calidad de la descendencia. Además, una gran disponibilidad de nutrientes también puede proporcionar más tiempo para el desarrollo adecuado de los embriones dentro de los huevos, en un lugar seguro. Las hembras de *L. schreiberi* con más carotenoides pueden invertir más antioxidantes en los huevos dando como resultado tiempos de incubación más largos. Aunque las hembras con coloraciones dorsales más verdes tenían huevos con menor tiempo de incubación, es probable que estos embriones tuvieran una tasa de desarrollo más rápida porque no mostraron diferencias de desarrollo.

La altura y el ancho de la cabeza de las crías estaban fuertemente influenciados por el tamaño de la cabeza de madre y la carga de hemoparásitos de la madre. Las madres con mayor intensidad de coloración UV en la garganta y coloración dorsal más saturada pero menos brillante (posiblemente debido a más melanina) tuvieron crías más grandes. Una coloración de la garganta UV-azulada más saturada pero menos brillante y una coloración del pecho amarillenta más saturada pero menos brillante de las madres pueden predecir una mejor condición corporal de las crías.

El tiempo en cautividad de las hembras preñadas afectó al éxito reproductivo y la coloración de las hembras. El tiempo en cautividad más largo resultó en menos crías y un éxito más bajo de eclosión, y las crías tenían tamaños de cuerpo medio y longitud y anchura de la cabeza más pequeños, y, además, estas crías tenían más anormalidades morfológicas. El tiempo en cautividad influyó negativamente en la saturación de color de las hembras, probablemente por la disminución del contenido de melanina y la reflectancia de los iridóforos de la piel. Este estrés probablemente aumentó el nivel de corticosterona en la sangre, lo que puede explicar el cambio de color de las madres y todos los efectos negativos sobre el éxito reproductivo, pero sorprendentemente, no afectó la condición de la madre ni a su respuesta inmune después de la puesta, a pesar de que la corticosterona es una hormona movilizadora de energía y que un aumento del nivel de corticosterona por el estrés crónico a largo plazo puede tener efectos supresores sobre el sistema inmune.

Conclusiones

- Con respecto a las estrategias antidepredatorias, el sexo o la condición reproductiva (estación de apareamiento) no afectan a la conducta preventiva de riesgo, ni a la decisión activa de escapar en una situación de emergencia. Por lo tanto, los machos no aplican compensación de comportamiento para el mayor riesgo de depredación que suponen la búsqueda de pareja y las señales sexuales conspicuas. Las estrategias antidepredatorias de *L. schreiberi* sólo dependen del entorno físico y la ubicación relativa del depredador y el refugio.
- La suplementación en la dieta de carotenoides es capaz de aumentar las señales visuales estructurales y basadas en carotenoides. La suplementación con carotenoides aumenta el contenido de carotenoides de los xantóforos, lo que contradice estudios anteriores y, además, fue capaz de cambiar el espaciamiento de las plaquetas reflectantes en los iridóforos.

- La suplementación de carotenoides no afecta a la respuesta inmune de los machos de L. schreiberi, al menos no a la respuesta inmune inducida por PHA. El desafío inmune inducido por LPS no tiene efectos sobre la coloración basada en carotenoides (probablemente luteína), ni tampoco muestra ninguna relación con la mayor parte del sistema inmune. Sin embargo, como las respuestas inmunes inducidas por PHA y LPS no cubren todo el sistema inmunológico, debemos tener cuidado con estas conclusiones. Sin embargo, los carotenoides tienen relación con la salud por medio de la condición corporal (almacén de grasa). La suplementación con carotenoides aumenta la condición corporal, mientras que cualquier efecto negativo en la condición corporal resulta en una disminución del croma de carotenoides en la coloración del pecho y la espalda, lo que sugiere que las señales basadas en carotenoides indican diferentes tipos de calidad que otras señales visuales.

- Los machos que sufrieron un desafío inmune aumentaron la intensidad de algunos componentes de las señales visuales, especialmente cuando se combinó con una suplementación de vitamina E en la dieta que puede contribuir a aumentar las señales sexuales, aumentando el contenido de vitamina E de las secreciones femorales y actuando como un antioxidante eficaz para sustituir el glutatión en las señales visuales basadas en melanina. Los machos con una activación inmune sufren un desafío relevante para el cuerpo y podrían estar tratando de maximizar su reproducción al aumentar la intensidad de la señal actual en una situación en la que las expectativas a largo plazo de supervivencia y reproducción futura son bajas. Por el contrario, el coste de aumentar la intensidad del color de la garganta parece ser, al menos en el tratamiento combinado, la disminución de carotenoides en otras partes del cuerpo que, sin embargo, parece ser menos importantes como señales sexuales en estos lagartos.

- La suplementación de la dieta con vitamina E resultó en un aumento consistente de vitamina E en las secreciones femorales en todos los tratamientos, pero encontramos

diferencias conspicuas al comparar los efectos de la suplementación con vitamina E en las señales visuales en dos experimentos diferentes sobre la misma población. La diferencia podría ser causada por las diferentes condiciones climáticas que supondrían diferentes desafíos para los machos al desarrollar señales sexuales, lo que podría ser la base de una elección de pareja por las hembras flexible. Por lo tanto, las señales multimodales de *L. schreiberi* pueden ser parte de un sistema especial de señales de respaldo. El contenido de vitamina E de las secreciones femorales puede ser una señal estable del estado antioxidante del macho, mientras que el estado de salud puede estar relacionado con una o más señales visuales influidas por el ambiente. Qué señal indica la calidad del macho dependerá de qué señal sea más fiable indicando calidad bajo cada condición ambiental determinada. Esto supondría la existencia de una elección de pareja flexible por las hembras que también puede ser la base de la evolución y mantenimiento de las señales múltiples de *L. schreiberi*.

- Las diferentes estrategias de uso del espacio pueden reflejar diferentes estrategias de apareamiento de los machos de *L. schreiberi*, a las cuales se adaptarían las hembras. Las diferentes estrategias de apareamiento de los machos pueden ser estrategias evolutivamente estables que proporcionan beneficios tanto para los machos que tienden a guardar parejas como para los machos dominantes. Un éxito reproductivo similar puede mantener la honestidad de las señales y, además, el sistema de señales sexuales múltiple en esta especie.

- Las hembras prefieren a los machos con mayor contenido de vitamina E en las secreciones femorales, lo que probablemente aumente la fertilidad. Nuestros resultados confirman que la vitamina E tiene un papel importante en el estado de salud, al menos en la respuesta inmune inducida por la PHA y el sistema antioxidante, lo que de hecho puede indicar la buena calidad de una pareja para las hembras.

- La coloración estructural, y las basadas en carotenoides y melanina de las hembras indican diferentes aspectos de su estado de salud y del número y calidad de su descendencia. Estas señales indicadoras apoyan la existencia de una elección de pareja mutua, especialmente en el caso de los machos con una estrategia de guarda de pareja, la cual, probablemente, puede tener un papel en la evolución y mantenimiento de la coloración de las hembras de *L. schreiberi*. Sin embargo, debido a que las bases estructurales y pigmentarias de la coloración en diferentes partes del cuerpo son similares en ambos sexos, no se excluye la existencia de correlación genética intersexual. Además, estos dos fenómenos aumentarían incluso sinérgicamente el fitness de los individuos que prefieren estas señales de color del otro sexo.

- Tanto las diferentes estrategias de uso del espacio y de apareamiento de los machos, como la elección flexible de pareja por las hembras dependiente del ambiente y la correlación genética intersexual pueden tener papeles en la evolución y el mantenimiento del sistema de señales múltiples sexuales de *L. schreiberi*.

Abstract

The main goal of this thesis is to study the function, evolution and maintenance of the multiple sexual signals that *Lacerta schreiberi* lizards use in sexual selection processes, and how the natural selection pressure on these signals affects antipredator strategies of lizards.

Conspicuous sexual signals, mate searching and mate guarding can greatly enhance the predation risk on males of sexually dichromatic species. Therefore, we investigated how sex, reproductive condition/mating season and the environment affect preventive risk-taking behavior (distance from the refuge), and active escape decisions in an emergency situation (flight initiation distance) of lizards (**Chapter I**). We found that males do not apply behavioral compensation for higher risk of mate searching and conspicuous sexual signals. In contrast, antipredator strategies of *L. schreiberi* only depend on the physical environment and the relative location of the predator and the refuge.

The elaboration of conspicuous sexual signals may have trade-offs with essential metabolic processes. We investigated the relationships among antioxidants, the immune system and multiple sexual signals of males **(Chapter II-IV).** We found that a carotenoid dietary supplementation is able to increase both carotenoid-based and structural visual signals. However, carotenoid supplementation does not increase the PHA-induced immune response, and, furthermore, an LPS-induced immune challenge neither has effects on carotenoid-based coloration. So, it seems that carotenoids do not boost immune system in this species. However, LPS and PHA-induced immune response do not cover the whole immune system. Nevertheless, carotenoids may have relation with health by increasing body condition (as fat stores). Carotenoid supplementation increases body condition, while a negative effect on body condition decreases carotenoid chroma in chest and back, suggesting that carotenoid signals indicate different types of quality than other types of visual signals.

Surprisingly, immune challenged males increase intensity of some visual signal components, especially when combined with supplementary dietary vitamin E, which can boost sexual signals, increasing vitamin E content of femoral secretion and acting as an effective antioxidant to substitute glutathione in melanin-based signals. Males with an immune activation suffer a relevant challenge for the body and might be trying to maximize future fitness by increasing current signal intensity in a situation where long term expectative of survival and future reproduction are low. In contrast, the price of the higher throat color intensity seems to be the carotenoid decreasing in other body parts that, nevertheless, seems to be less important as sexual signals in these lizards. Furthermore, we found that vitamin E supplementation enhances PHA-induced immune response, shows a consistent increase in vitamin E content in femoral secretion in all treatments. However, we found conspicuous differences when comparing vitamin E supplementation effects on visual signals in two different experiments on the same population made in different years. Differences might be caused by different weather conditions with different challenges of males when developing sexual signals, which might be, however, the base of flexible female choice. Therefore, multimodal signals of *L. schreiberi* can be part of a special back-up signal system. Vitamin E content of femoral secretion may be a stable signal about the antioxidant status, while simultaneously health status might be related to one or more environmentally influenced visual signals. Which signal indicates male quality would depend on which visual trait is more reliable showing quality under each given environmental conditions. This alteration in male sexual signals would suppose the existence of flexible female mate choice that can also be the base of evolution and maintenance of multiple signals of *L. schreiberi*.

Movement patterns of animals can reveal social relationships and mating behavior of a species, which can show the function of signals and strongly influence their evolution and maintenance. Therefore, we examined home range size and intra and interspecific overlappings of home ranges in *L. schreiberi* (**Chapter V**). We found that dominant males use larger home ranges that overlap with more and more dominant males, and with more females, likely trying to increase reproductive success by mating with a higher quantity of females. While less dominant males use small home ranges, and prefer mate guarding on

less females but of higher quality of. Females using larger home ranges, overlap with more males, but females overlapping males with high α-tocopherol content in chemical signals (i.e. of high quality) use small home range areas. Different mating strategies of males may be evolutionary stable strategies which provide benefits both to mate guarder and dominant males. Similar reproductive success of different strategies could maintain honesty of signals and, furthermore, the multiple sexual signal system of this species.

Researching the function, evolution and maintenance of female coloration (**Chapter VI**), we found that structural, carotenoid and melanin-based coloration of females together may indicate their health state and their offspring number and quality. These indicator signals support the existence of mutual mate choice, especially in case of males with a mate guarding strategy, which, likely, may have a role in the evolution and maintenance of *L. schreiberi* female coloration. However, due to the similar pigment and structural bases of coloration in different body parts in both gender, intersexual genetic correlation is neither excluded. Moreover, these two phenomena would increase even synergistically the fitness of individuals that prefer partners with these color signals.

In summary, we found that both the different mating strategies of males, the environment-dependent flexible mate-choice and the intersexual genetic correlation may have roles in the function, evolution and maintenance of multiple sexual signals in *L. schreiberi*.

Resumen

El objetivo principal de la tesis es estudiar la función, evolución y mantenimiento de las señales sexuales múltiples que utilizan el lagarto verdinegro (*Lacerta schreiberi*) en procesos de selección sexual, y cómo la presión de la selección natural sobre estas señales afecta a las estrategias antipredatorias.

Capítulo I. Se investigó si el sexo, condición reproductora y el ambiente afectan al comportamiento de escape. Encontramos que los machos no compensan un mayor riesgo debido a la búsqueda de pareja y señales sexuales conspicuas. Por el contrario, las estrategias antipredatorias sólo dependen del entorno físico y la ubicación relativa del depredador y el refugio.

Capítulos II-IV. Se investigó las relaciones entre los antioxidantes, el sistema inmunológico y múltiples señales sexuales de los machos. Encontramos que:

• La suplementación en la dieta de carotenoides aumenta las señales visuales, pero no estimulan el sistema inmune (respuesta inmune inducida por LPS y PHA). Aunque estos dos métodos no cubren todo el sistema inmune. Sin embargo, los carotenoides pueden tener relación con la salud aumentando la condición corporal.

• Los machos que sufren una activación inmune aumentan la intensidad de algunas señales visuales, especialmente cuando tienen suplementos en la dieta de vitamina E. Estos machos sufren un desafío relevante y podrían estar tratando de maximizar el fitness aumentando la intensidad de la señal actual puesto que las expectativas a largo plazo de supervivencia y reproducción futura son bajas.

• La suplementación con vitamina E aumenta la respuesta inmune inducida por PHA, y, además, resulta en un aumento del contenido de vitamina E en las señales químicas.

• Existen diferencias al comparar los efectos de la suplementación de vitamina E en las señales visuales en dos experimentos en la misma población realizada en diferentes años. Esto puede ser causado por diferentes condiciones climáticas que suponen diferentes desafíos para los machos cuando desarrollan las señales. El contenido de vitamina E en las secreciones femorales puede ser una señal estable del estado antioxidante, mientras que el estado de salud puede estar relacionado con una o más señales visuales influenciadas ambientalmente. Qué señal indica la calidad del macho dependerá de qué señal sea más fiable indicando calidad bajo cada condición ambiental. Esto supondría la existencia de una elección de pareja flexible por las hembras que también puede ser la base de la evolución y mantenimiento de señales múltiples de *L. schreiberi*.

Capítulo V. Se examinó si el tamaño del área de campeo y el solapamiento entre áreas en *L. schreiberi* pueden revelar sus relaciones sociales y comportamiento de apareamiento. Encontramos que los machos dominantes usan áreas más grandes, probablemente tratando de aumentar el éxito reproductivo al solapar y aparearse con más hembras. Los machos menos dominantes usan áreas más pequeñas y prefieren guardar parejas, accediendo a menos hembras pero de mayor calidad. Las hembras que utilizan áreas más grandes solapan con más machos, pero las hembras que solapan con machos con más vitamina E en sus señales químicas (de alta calidad) usan áreas más pequeñas. Las diferentes estrategias de apareamiento de los machos pueden ser estrategias evolutivamente estables que proporcionan beneficios para ambos tipos de machos. Un éxito reproductivo similar podría mantener la honestidad de las señales y el sistema de señales sexuales múltiples.

Capítulo VI. Se investigó la función de la coloración de las hembras de *L. schreiberi*. Encontramos que esta coloración puede indicar su estado de salud y el número y calidad de sus crías. Estas señales indicadoras apoyan la existencia de una

elección de pareja mutua, la cual puede tener un papel en la evolución de la coloración en hembras. Sin embargo, debido a que las bases de la coloración son similares en ambos sexos, no se excluye la correlación genética intersexual.

Se concluye que tanto las diferentes estrategias de apareamiento de los machos, la elección de pareja flexible dependiente del ambiente y la correlación genética intersexual pueden tener papeles en la función, evolución y mantenimiento de múltiples señales sexuales en *L. schreiberi*.